DISCOVERY OF NOVEL EFFECTORS OF THE PROTEASOME PATHWAY: CYCLOPENTENONES AS INHIBITORS OF UBIQUITIN ISOPEPTIDASE ACTIVITY

by

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ABSTRACT

I discovered that certain electrophilic prostaglandins inhibit the ubiquitin-specific protease (USP) activity of the proteasome pathway. Herein, evidence is presented that supports the hypothesis that the cross-conjugated α,β-unsaturated dienone is a molecular determinant for the potency of this activity, and that this chemical feature causes an alteration in cellular ubiquitin dynamics, resulting in decreased free ubiquitin and decreased protein degradation. I show that this decrease in protein degradation activates the unfolded-protein response (UPR) of both the cytoplasm and the endoplasmic reticulum, likely due to the accumulation of deranged/misfolded proteins. I make the novel observation that as an attempt to compensate for the loss in protein degradation by the proteasome pathway, the lysosomal degradation pathway is activated in USP inhibitor treated cells. Lastly I show that, ultimately, cell death occurs due to the build-up of toxic levels of cellular protein. These data reconcile previously known effects of prostaglandin treatment, namely that heat-shock proteins are up-regulated and that a number of short-lived proteins are stabilized, and in so doing, establish a cohesive model for prostaglandin-induced apoptosis. The potential that components of the ubiquitin-proteasome pathway may be useful targets for cancer chemotherapy has been realized only recently with the success, in human clinical trials, of the proteasome

inhibitor, VELCADE^{TM} (PS-341). Given that several hundred other potential molecular targets reside within the proteasome pathway, there is intense interest in discovering novel points for drug intervention. Our data suggest that inhibition of USP activity represents a legitimate target for chemotherapeutic development.

This thesis is dedicated to my mother and father, whose continuous love and support were essential to my maturation as a person and a student. This thesis is also dedicated to my mentor, Dr. Frank Fitzpatrick, Ph.D., whose untiring efforts to further my development as a scientist and to promote my work have made a lasting contribution to my future success.

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CHAPTER 1

INTRODUCTION: UBIQUITIN-PROTEASOME PATHWAY IN CANCER

1.1. Overview of Ubiquitin-Proteasome Pathway

The ubiquitin-proteasome pathway is responsible for the degradation of 70 to 90 percent of all cellular proteins [1]. As the principal means for cellular protein degradation, this pathway has two primary functions: a regulatory function (i.e., the targeted degradation of functionally competent proteins) and a quality control function (i.e., the degradation of misfolded, functionally incompetent proteins to prevent protein aggregation) [2,3]. Underscoring its importance to cellular homeostasis, the ubiquitin-proteasome pathway is present in all eukaryotes and many of its components are highly conserved [4]. Owing to the recent clinical success of the proteasome inhibitor, VELCADE™ (PS-341), the components of this pathway have generated a great deal of interest as targets for cancer chemotherapy. The focus of this chapter is to describe, in some detail, the components of the ubiquitin-proteasome pathway, the major processes controlled by this pathway, known derangements in the pathway that may lead to carcinogenesis, how inhibitors of this pathway may cause preferential cancer cell death, and preliminary results from the clinical trials of VELCADE™.

1.1.1. Ubiquitin-Proteasome Pathway Components

The ubiquitin-proteasome pathway (Figure 1.1) is comprised of four major elements: a protein that serves as a signal for the degradation of targeted proteins, enzymes that covalently attach that signal to the targeted proteins, the proteolytic complex that degrades the targeted proteins while sparing the signaling protein, and the group of enzymes responsible for recycling the signaling protein so that it

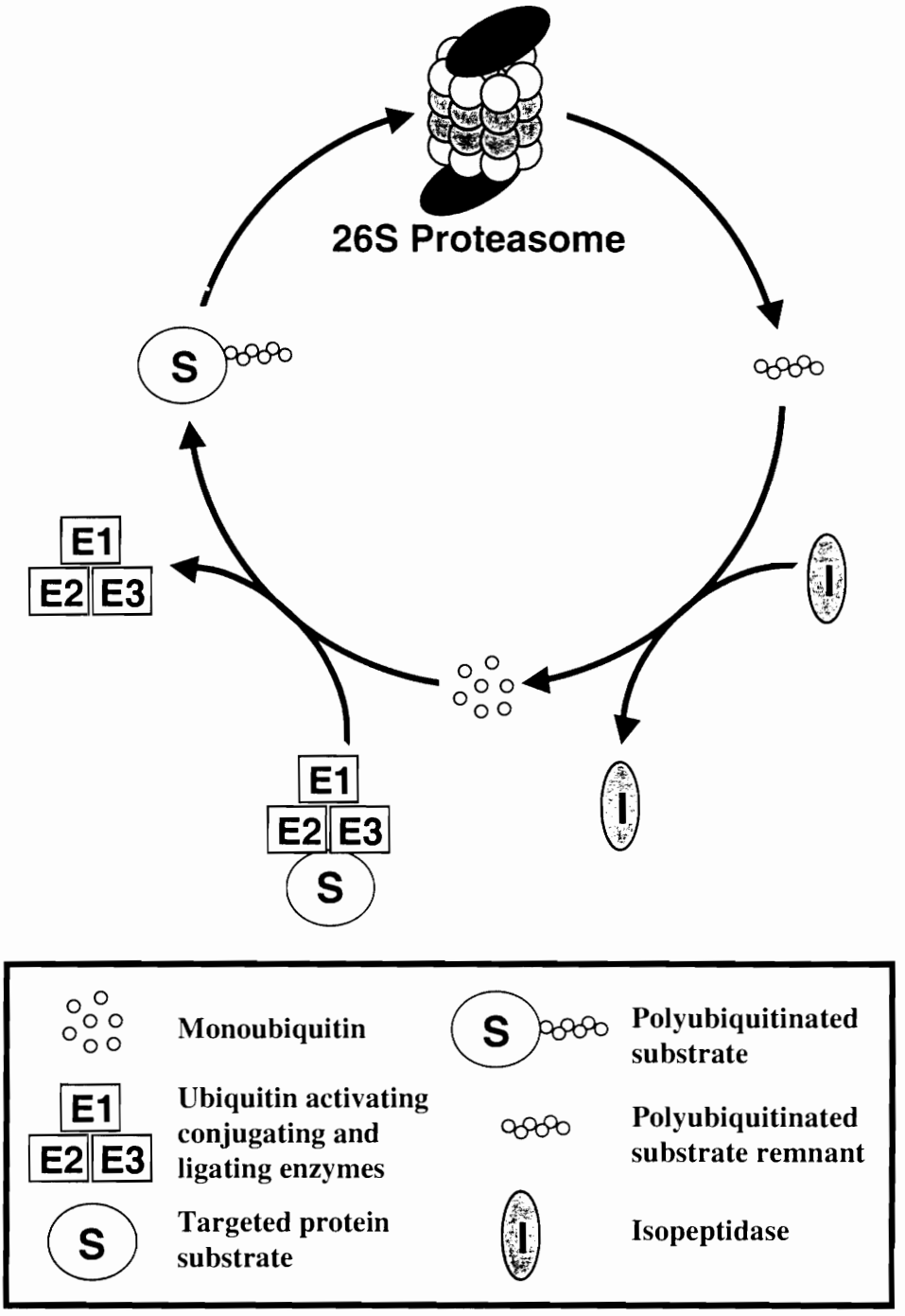


Figure 1.1. The Ubiquitin-Proteasome Pathway. Schematic depicts the ubiquitination and degradation of a targeted protein by the ubiquitin-proteasome pathway. The cyclic nature of the pathway is derived from the perspective of ubiquitin.

may be used in another round of targeting. Together, these elements form the basis for the primary means by which all eukaryotic cells degrade intracellular proteins [1]. The proteasome also plays an important role in antigen presentation, though this aspect will not be addressed in this work.

1.1.1.1. Ubiquitin

The protein that can serve as a signal for proteasome-mediated degradation is ubiquitin. Ubiquitin is present in all eukaryotes, and is expressed in every cell type that has been examined, at concentrations approaching 1 μM. It is comprised of 76 amino acids, the sequence of which is highly conserved from yeast to humans (96 percent identity in primary structure), making it one of the most highly conserved eukaryotic proteins known [5]. Three classes of genes have been identified that, when expressed, produce ubiquitin; all of the ubiquitin gene products result in ubiquitin fusion proteins, in which the carboxyl-terminus of ubiquitin is fused to the amino-terminus of another protein, and therefore must be post-translationally processed to release monoubiquitin [6,7]. The translational products of the two UBA genes are single ubiquitin units fused to the amino-termini of the small ribosomal proteins, s27a and L40; this fusion is believed to aid in the proper folding of the ribosomal proteins [8]. The translational products UBB and UBC genes are fused multimers of ubiquitin, with the UBB gene consisting of three ubiquitin units and the UBC genes consisting of up to seven ubiquitin units [6].

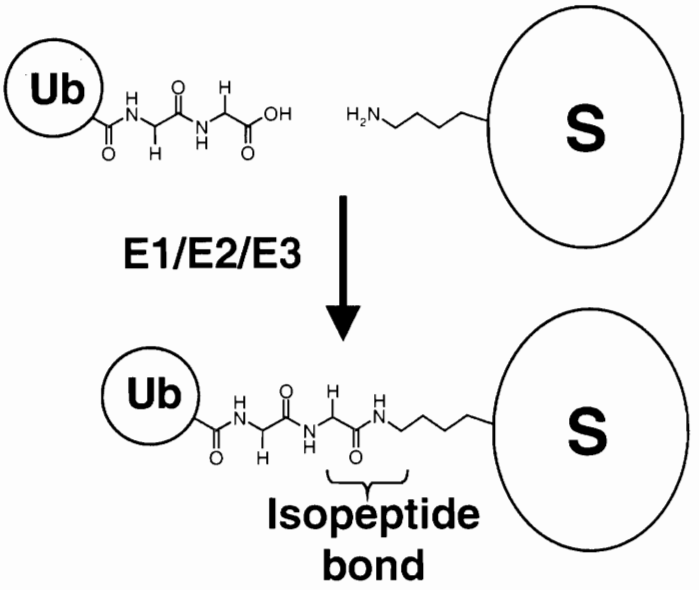
In a process that will be described below, either a ubiquitin monomer (monoubiquitination) or a ubiquitin polymer (polyubiquitination) can be covalently bound to a targeted protein (Figure 1.2). Unlike the peptide bond-linked ubiquitin fusion proteins that are the products of the ubiquitin genes, monoubiquitination results in the formation of an isopeptide bond [9]. The isopeptide bond is a covalent bond between the carboxyl-terminus of ubiquitin and the ε-amino group of a lysine on the targeted protein. In the case of polyubiquitination, a targeted protein substrate is first monoubiquitinated on a lysine residue via an isopeptide bond. Then, ubiquitin monomers are added sequentially, via isopeptide bonds, to lysine residues on the preceding ubiquitin. Alternatively, preassembled polyubiquitin can be conjugated to targeted proteins [10,11]. The end of the polyubiquitin chain that is attached to the target substrate is termed the proximal end.

1.1.1.2. Ubiquitin Activating, Conjugating, and Ligating Enzymes

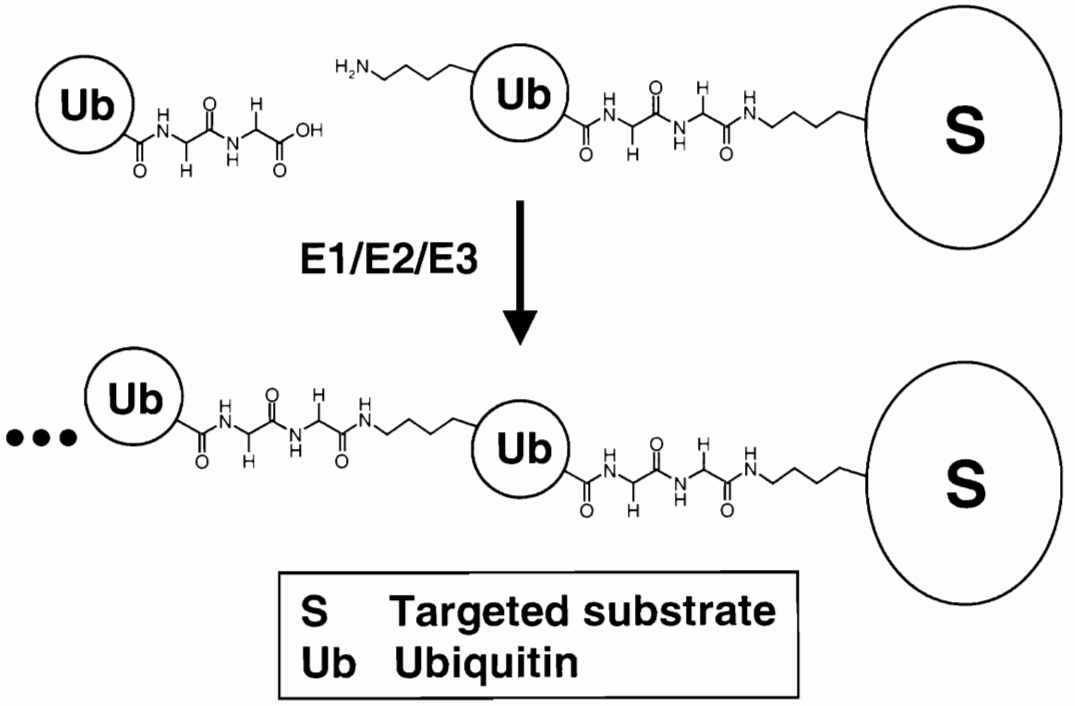
Three enzyme families play a part in the ubiquitination of targeted proteins: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes (or ubiquitin-carrier enzyme), and E3 ubiquitin-ligating enzymes [12]. There are only a few known E1 enzymes, approximately 50 E2 enzymes, and as many as 1000 E3 enzymes. E1 ubiquitin-activating enzymes function, in an ATP-dependent manner, to form a high-energy thioester bond between the carboxyl-terminus of ubiquitin and a cysteine sulfhydryl group in the E1 active site [13]. This high-energy bond is used to transfer ubiquitin to E2 ubiquitin-conjugating enzymes,

Figure 1.2. The Isopeptide Bond. Schematic depicts the monoubiquitination and polyubiquitination of a targeted substrate. Chemical structures show the lysine side chains for a targeted substrate (monoubiquitination) and for ubiquitin (polyubiquitination), the C-terminal glycines of ubiquitin, and the resulting isopeptide bond.

Monoubiquitination



Polyubiquitination



S	Targeted substrate
Ub	Ubiquitin

which functions as a carrier protein and works in concert with the E3 ligases [12]. Depending on the type of E3 ligase involved (HECT, RING finger, PhD, or U-box domain ligases), the ubiquitin bound to the E2 ubiquitin-conjugating enzyme can either be directly conjugated to the targeted protein (RING finger ligases), or first transferred to an E3 ligase (HECT domain ligases), which via another thioester bond, conjugates ubiquitin to the targeted protein [14,15]. Of these three major enzyme families involved in ubiquitination, it is the E3 ligases that appear to confer substrate specificity to the ubiquitin-dependent pathways.

Although much remains to be discovered about the properties conferred by monoubiquitination, several effects have been demonstrated. As has been shown with the ubiquitin interacting motifs (UIMs) of the endocytic proteins, Eps15 and eps15R, monoubiquitination can result in changes in protein-protein interactions by serving as a removable targeting domain [16]. Also, monoubiquitination can result in endocytosis and lysosomal degradation of membrane bound proteins, such as epidermal growth factor receptor (EGFR) [17]. Lastly, as will be discussed in more detail in section 1.1.2.3., monoubiquitination of histones in yeast has effects on gene transcription [18].

Like monoubiquitination, much remains to be discovered about the varied effects conferred by polyubiquitination of targeted proteins. However, the primary reason for these varied effects is related to the way in which the isopeptide linkages in the polyubiquitin chain are assembled. There are multiple lysines on ubiquitin that can be utilized to form polyubiquitin chains, lysine 11 (K11), K29, K48, and K63 [19].

K48-linked polyubiquitin chains, the best characterized of the chain linkages, target proteins for proteasomal degradation by serving as a recognition signal by the 26S proteasome [20]. In order for a protein to be targeted for proteasomal degradation, a polyubiquitin chain consisting of four or more K48-linked ubiquitin subunits must be attached [21]. As mentioned, this can be accomplished by covalent modification with a preassembled polyubiquitin chain or by the sequential addition of ubiquitin monomers.

Unlike the K48-linked chain, the K63-linked polyubiquitin chain does not appear to target proteins for proteasome-mediated degradation. As has been suggested with K63-linked polyubiquitination of proliferating cell nuclear antigen (PCNA), in some cases this modification may serve as a signal or framework for the assembly of enzymatic complexes (see section 1.1.2.4.) [22]. The ubiquitin ligase, Traf6, catalyzes the formation of K63-linked chains. Conjugation of K63-linked chains by Traf6 has been shown to activate protein kinases of the NFκB pathway, TAK1 and IκB kinase (IKK), and the Jun N-terminal kinase pathway, JNK/p38 [23,24]. Finally, K63-linked chains appear to play an essential role in the nonproteasomal degradation of membrane proteins such as Gap1, whereby this type of polyubiquitin chain acts as a signal for endocytosis [25].

Recent evidence has suggested a role for the K11-linked polyubiquitin in regulating the localization of the chaperone recruiting protein, BAG-1 [26]. This BAG-1 modification appears to be mediated by the heat-shock protein (HSP) associated ubiquitin ligase, CHIP, targeting CHIP to the proteasome. Remarkably, proteasomal targeting of CHIP does not target it for degradation. The function of

CHIP on the proteasome appears to be related to the recruitment of HSPs, presumably so that they may deliver protein substrates for degradation [26].

No function has of yet been assigned to the K29 linkage, but the formation of this linkage has been observed in rabbit reticulocyte lysate as well as in yeast, suggesting it plays some physiological role [27,28]. Therefore, there is still much to learn about all of the effects polyubiquitination can have on substrates.

1.1.1.3. The 26S Proteasome

The next component of the ubiquitin-proteasome pathway consists of the approximately two megadalton 26S proteasome, which is tasked with the degradation of polyubiquitinated substrate proteins [20]. In vertebrates, the core of this complex is the 20S catalytic subunit, which is made up of four concentric rings: two identical adjacent inner rings, consisting of seven different β-subunits each, and two identical outer rings, consisting of seven different α-subunits each (Figure 1.3) [29]. In addition, three γ-interferon-inducible β-subunits can be expressed that substitute for the constitutively expressed β-subunits [30,31]. The β-subunits are threonine proteases that possess all of the proteolytic activity of the complex, however, in eukaryotes, only three of the seven subunits appear to be active [32]. The outer α-subunits appear to serve two main functions: as a docking site for the 19S regulatory subunit, and as a regulatory mechanism that prevents the entry of substrates into the catalytic chamber in the absence of the 19S regulatory subunit [33].

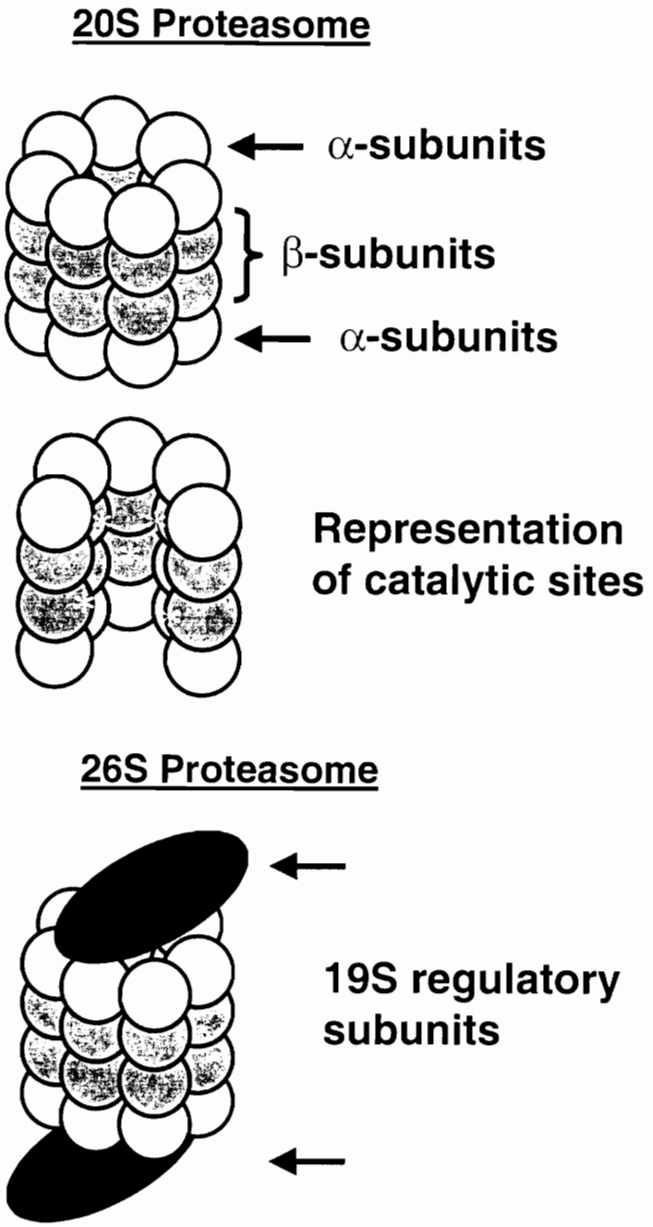


Figure 1.3. The Proteasome Components. Schematic depicts the 20S proteasome core, a cross-section of the core particle, and the 26S proteasome holoenzyme.

The 19S regulatory subunit is made up of at least 18 proteins and functions as a recognition element for the polyubiquitin degradation signal, and as an ATP dependent protein-substrate unfolding complex [34]. It is the 19S regulatory subunit that recognizes and binds polyubiquitinated substrates. The requirement for this binding is that the targeted substrates have a polyubiquitin chain of at least four ubiquitin units attached, and that the chain is of the K48-linkage type [21]. These requirements, as well as the necessity of protein-substrate unfolding in order to access the catalytic chamber, are what prevent the proteasome from performing indiscriminant protein degradation.

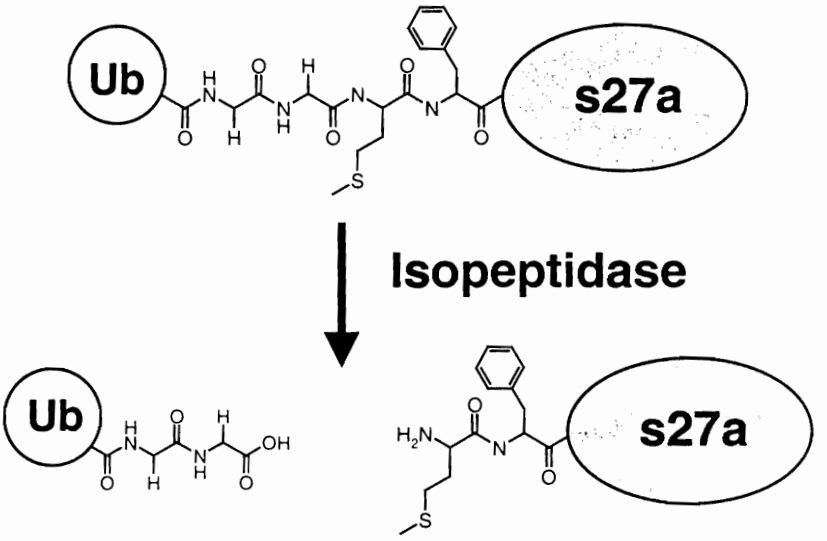
1.1.1.4. Ubiquitin-Specific Proteases (Ubiquitin Isopeptidases)

The final family of enzymes in the ubiquitin-proteasome pathway is the ubiquitin-specific protease (USPs) family. These enzymes are also termed isopeptidases (the term used throughout this thesis). This family is comprised of as many as 90 members, most of which are cysteine proteases [35,36]. They are broken down into three major types according to protein sequence homology: ubiquitin C-terminal hydrolases (UCHs), ubiquitin processing proteases (UBPs), and a newly identified class, the metalloproteases [37].

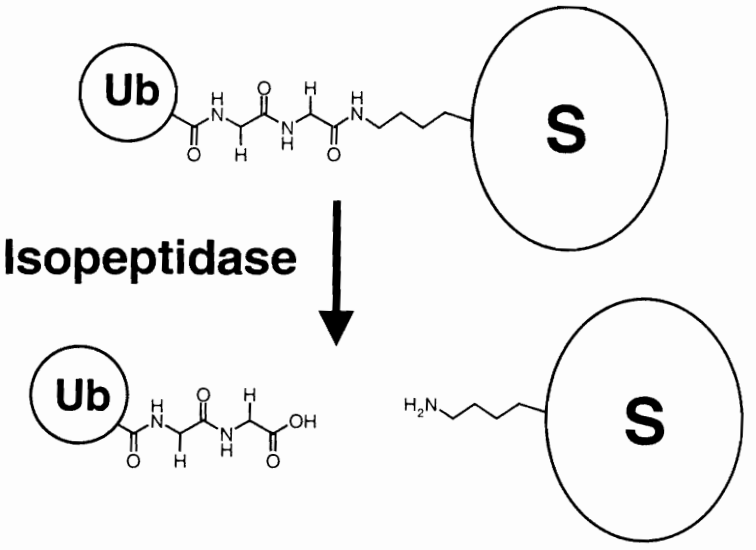
Isopeptidases have in common the ability to hydrolyze peptide or isopeptide bonds between the C-terminus of ubiquitin and another protein (Figure 1.4). Therefore, the various members of this enzymatic class can process ubiquitin fusion proteins (products of the ubiquitin genes), remove small peptide remnants present on the proximal end of polyubiquitin following proteasomal degradation,

Figure 1.4. Isopeptidase Substrates. Schematic depicts the isopeptidase-mediated hydrolysis of a linear ubiquitin fusion protein (peptide bond hydrolysis) and a monoubiquitinated protein (isopeptide bond hydrolysis).

Hydrolysis of ubiquitin fusion proteins



Hydrolysis of isopeptide bonds



S	Targeted substrate or ubiquitin
Ub	Ubiquitin
s27a	Ribosomal protein s27a

edit improperly polyubiquitinated protein substrates, and rescue targeted protein substrates from proteasomal degradation [35].

The UCHs are the smallest of the isopeptidase family, with molecular weights of less than ~40 kilodaltons. UCHs contain Cys and His boxes, characteristic secondary structure elements of the cysteine protease class of isopeptidases that make up the catalytic core of these enzymes [38]. As is common with the isopeptidase family, the regulation, tissue-specific expression, and substrate specificity of the UCHs is largely unknown. Of this class, only the brain-specific UCH-L1 (Protein Gene Product 9.5, PGP 9.5) has been characterized as having tissue specific expression [39]. Interestingly, this isopeptidase is overexpressed in a number of cancers and cancer cell lines, though its role in this regard has not been elucidated [40,41]. The only UCH having a proposed function is UCH-L5, a 19S regulatory cap isopeptidase that appears to play an essential role in proteasomal degradation [42].

The UBPs are the largest of the catalytic cysteine-containing ubiquitin protease class, having molecular weights ranging from 50 to 250 kilodaltons. UBPs share in common with the UCHs the Cys and His boxes, however the remainder of the sequences of these two classes are largely divergent, as they most likely play a role in regulation or substrate recognition of the individual isopeptidases [35]. Like the UCHs, little is known about the substrate specificity of the individual UBPs, with two exceptions; isopeptidase-T (USP5) appears to be primarily responsible for the disassembly of polyubiquitin, starting from the proximal end, and USP16 appears to deubiquitinate the monoubiquitinated

histones, H2A and H2B [43,44]. Given that ubiquitin is ligated to so many diverse substrates, the role of many of the remaining isopeptidases is likely to be in the regulation of ubiquitination of these proteins. In addition, some of these isopeptidases may play a role in the removal of some of the approximately 11 ubiquitin-like proteins (e.g., Nedd8, Sumo1, etc.) [45].

The most recently identified family of ubiquitin specific proteases is the metalloprotease family. During ubiquitin-mediated proteasomal degradation, the polyubiquitin degradation signal is spared the fate of the targeted protein via the actions of isopeptidases resident in the 19S regulatory subunit, which release polyubiquitin from the complex. One of these isopeptidases has recently been identified as the zinc protease, POH1 [37]. It shares no protein sequence homology to the known cysteine protease isopeptidases; however it has significant homology to JAB1, a protein known as an isopeptidase for the ubiquitin-like protein, Nedd8 [46].

1.1.2. Substrates of the Ubiquitin-Proteasome Pathway and Cellular Processes Controlled

As mentioned, the proteasome is responsible for the degradation of 70 to 90 percent of proteins in the cell. It is therefore easy to understand that the proteasome pathway in some way modulates most cellular processes (e.g., cell cycle regulation, apoptosis, and transcription, etc.). The importance in understanding the implications of modulation of these processes becomes evident when considering both the diseases that arise from aberrant proteasomal

degradation as well as the therapeutic manipulation of the ubiquitin-proteasome pathway.

1.1.2.1. The Cell Cycle

Cyclin-dependent kinases (CDKs) are the primary effector molecules that regulate the cell cycle [47]. CDKs are constitutively expressed throughout the cell cycle, so their activity must be modulated in order for proper cell cycle balance to be maintained. This activity is modulated via association with two classes of regulatory molecules: cyclins (e.g., cyclins A, B, D, and E) and cyclin-dependent kinase inhibitors (CDKIs; e.g., $p21^{Cip1}$ and $p27^{Kip1}$) [47,48]. Cyclin binding activates CDKs while CDKI binding inhibits activation of CDKs. It is the fluctuations in cyclin and CDKI protein levels that modulate CDKs, and therefore the cell cycle.

Two large complexes play a dominant role in regulating cyclin and CKDI protein levels; the SCF ubiquitin ligase complex, so called because it is composed of the Skp1, Cullin, and F-box proteins, and the anaphase-promoting complex (APC), which is composed of a Cullin homolog and several other proteins unrelated to those of the SCF complex [49]. The F-box proteins of the SCF complex are variable subunits that allow for the targeting of diverse substrates for ubiquitination [50]. The APC complex is believed to have variable targeting subunits similar to F-box proteins. The APC complex targets cyclins A and B for ubiquitination, while the SCF complex targets cyclin E and the CDKI, p27 [49]. Although the regulation of this process is considerably more complex than

discussed here, clearly the ubiquitin-proteasome pathway plays a major role in cell cycle regulation.

1.1.2.2. Apoptosis

Analogous to the balance maintained with the components of the cell cycle machinery, the proteasome pathway is also essential in maintaining control of the balance between life and death of the cell. As this balance is tilted toward programmed cell death, or apoptosis, events lead to the activation of the effector molecules of cell death, caspases [51]. Caspases reside in cells in a pro-apoptotic form, awaiting signals leading to their cleavage and activation, which in turn leads to the cleavage and activation of other caspases in a cascade resulting in cell death. In order to prevent premature initiation of caspase cascades, inhibitor of apoptosis proteins (IAPs) bind to activated caspases, preventing their ability to initiate the cell death cascade [52].

Some IAPs (e.g. XIAP, c-IAP1, and c-IAP2) are E3 ubiquitin ligases, the significance of which is not yet completely understood [52]. However, one identified function of this E3 ligase activity is for IAP autoubiquitination [53]. In fact, experiments have shown that wild type E3 ligase IAP protein levels decrease during apoptosis, while generated mutant IAPs lacking the ring finger motif essential for ubiquitination are relatively stable and prevent apoptosis [53]. This suggests that autoubiquitination of IAPs is activated upon induction of apoptosis, resulting in their degradation and subsequent initiation of caspase cascades and cell death.

1.1.2.3. Transcription

Transcription factors are important regulators of processes as diverse as cellular homeostasis, differentiation, and oncogenic transformation. Many tumor suppressors are transcription factors whose cellular protein levels, and therefore activity, are regulated by proteasomal degradation [54]. The p53 tumor suppressor is one such transcription factor, the intracellular levels of which are kept low, under basal conditions, by the actions of the E3 ubiquitin ligase, human double-minute 2 (HDM2) [55,56]. Upon sensing genomic damage, or other indicators of cellular stress, posttranslational modifications to both p53 and HDM2 prevent their association, and p53 ubiquitination, allowing for the intracellular accumulation of p53 so that it may act as a sentinel of genomic integrity [57].

Nuclear factor kB (NFκB) is a transcription factor whose activity is regulated *indirectly* by proteasomal degradation. Under basal conditions NFκB is inhibited by its association with the protein, inhibitor of κB (IκB) [58]. IκB functions as an inhibitor of NFκB by binding to, and thus masking, the NFκB nuclear localization signal. Upon activation of cell surface receptors, a chain of events unfolds, leading to the activation of the serine/threonine kinase, IκB kinase (IKK), which phosphorylates IκB, causing it to dissociate from NFκB and be targeted for proteasomal degradation [58]. This action exposes the NFκB nuclear localization signal, allowing for nuclear import of NFκB and activation of NFκB target genes.

The ubiquitin-proteasome pathway also regulates transcription via ubiquitination of the transcriptional activation domain (TAD) present on many

transcription factors [59]. Interestingly, the TAD domain serves not only as an activator of transcription, but also as the proteasome-mediated degradation signal, or degron, of these transcription factors. As such, the TAD domain requires ubiquitination for both its activation and degradation. Like other substrates for ubiquitination, TAD domains appear to be selectively ubiquitinated, thus allowing for the discreet regulation of individual transcription factors and subsequent target gene families.

Finally, ubiquitination can regulate transcription in a more general way, via ubiquitination of histones [18]. Histones are proteins that serve as a structural/regulatory core around which DNA is wrapped to form chromatin [60]. Histones are the most abundant ubiquitinated cellular proteins and were once believed to merely serve as a pool of monoubiquitin that could be tapped during times of cellular stress. However, ubiquitin already exists in the cell as a large pool of monoubiquitin, leading others to hypothesize that a more significant role for histone monoubiquitination may involve DNA function.

The current model for DNA transcription suggests that basic lysine residues on the N-termini of histones cause DNA to tightly bind these molecules, thereby leading to gene repression [61]. Neutralization of the positive charge on one or more of these lysines, via acetylation or methylation, results in the local unwinding of the histone-DNA complexes, allowing for local gene transcription. Recent evidence in yeast has demonstrated that methylation of histone H3 (on K4) is necessitated by the monoubiquitination of histone H2B (on K123) by the

ubiquitin conjugating enzyme, Rad6, thereby providing a mechanistic link between ubiquitination and gene transcription [18,62].

1.1.2.4. DNA Repair

Another role of the ubiquitin-proteasome pathway that has been recently discovered is that of DNA repair [22]. Proliferating cell nuclear antigen (PCNA) is a protein known to be involved in DNA synthesis and repair [63]. Work in yeast has demonstrated how ubiquitin plays a role in error-free DNA repair. This type of repair, dependent on DNA replication, uses the undamaged sister duplex as a template to replace a damaged base [64,65]. When error-free DNA repair is not available, error-prone DNA repair takes place by default, using translesion polymerases to replace the damaged base with any of the four bases [66]. The latter repair method can obviously result in DNA mutations leading to the production of aberrant proteins. The model generated by this recent work in yeast suggests that the Rad6 ubiquitin-conjugating enzyme associates with the Rad18 ubiquitin ligase, resulting in monoubiquitination of PCNA [22]. Rad6/Rad18 can then form a larger complex with the Ubc13 ubiquitin conjugating enzyme and the Rad5 ubiquitin ligase to form a lysine-63 linked polyubiquitin chain on the previously ligated ubiquitin. As mentioned previously, the K63 linkage in polyubiquitin does not generally lead to degradation, as does K48-linked polyubiquitin. As anticipated, mutation of the ubiquitinated lysine (K164R) of PCNA led to sensitivity to DNA damaging agents [22].

Yeast PCNA shares 35 percent identity in its primary structure with human PCNA, and importantly, lysine 164 is conserved [63]. Ubiquitinated PCNA was not observed in untreated HeLa cells. However, when these cells were treated with a DNA damaging agent, polyubiquitinated PCNA was observed [22]. If this polyubiquitinated PCNA is K63-linked, this will demonstrate the first link in humans between ubiquitination and DNA repair processes.

1.2. Derangements in Proteasomal Degradation That Are Implicated in Carcinogenesis

Given that the ubiquitin-proteasome pathway plays such a prominent role in cellular protein degradation, and that the activity of many proteins is proportional to their intracellular concentration, it is not surprising that many diseases can arise due to the aberrant degradation of particular substrates. Accordingly, alterations in proteasomal degradation have been linked to carcinogenesis. Discussed in this section are just a few of the many examples of dysregulated proteins and the proposed mechanisms regarding their role in carcinogenesis.

1.2.1. Human Papillomavirus E6 Oncoprotein

Approximately 200,000 women die worldwide from cervical cancer every year. Most cases of cervical cancer have been associated with the presence of the human papillomavirus E6 (HPV-E6) oncoprotein [67]. This oncoprotein forms a complex in cells with an endogenous host protein, E6-associated protein (E6-AP).

Analogous to the endogenous p53-specific E3 ubiquitin ligase, HDM2, the HPV-E6/E6-AP complex functions as an E3 ubiquitin ligase for the p53 tumor suppressor, resulting in decreased basal levels of p53 protein [68]. However, unlike HDM2, the E6 oncoprotein is not itself regulated upon cellular exposure to DNA damage or cellular stress, therefore it continues to act in conjunction with E6-AP to prevent the accumulation of p53, resulting in decreased genetic surveillance. It is believed that this chronic suppression of p53 leads to the accumulation of secondary genetic mutations, some of which may eventually lead to cancer progression.

1.2.2. NFκB Pathway

The increase in tumor burden that is the phenotypic hallmark of cancer is most often the result of increased cellular proliferation. However, a phenomenon that can have equally important implications, both in terms of cancer mechanism and in terms of how a particular cancer can be most successfully treated, is decreased cell death. As discussed above, NFκB is a transcription factor that is indirectly regulated by the ubiquitin-proteasome pathway. The expression of genes that are regulated by NFκB, such as IAPs (section 1.1.2.2.), can result in increased cell survival [69,70]. Also, NFκB activation can induce the expression of anti-apoptotic Bcl2 family members, resulting in increased resistance to chemotherapeutic agents [71]. Furthermore, overexpression of either IKK or NFκB is sufficient to result in the transformation of cancer cells [72,73]. Although no definitive mutations have been identified linking aberrant ubiquitination of its

inhibitor, IκB, with carcinogenesis, increased levels of NFκB and its activator, IKK, have been observed in cancer cell lines [74].

1.2.3. β-Catenin

β-Catenin has two primary functions in cells; as an adherens junction protein, it plays a role in cell adhesion, and as a binding partner of T cell factor (Tcf) and lymphoid enhancer factor (Lef) transcription factors, it facilitates transcription of *Wnt* signaling pathway genes [75]. Up-regulated β-catenin has been linked to the pre-cancerous syndrome, familial adenomatous polyposis of the colon (FAP) [76]. The primary genetic defect in this syndrome resides within the *APC* gene, the product of which (APC) interacts with and down-regulates β-catenin, and thus *Wnt* signaling [77]. APC down-regulates β-catenin by acting as a co-factor, with the serine-threonine kinase GSK-3β, in the phosphorylation of β-catenin [78]. This phosphorylation allows for the recognition of β-catenin by the F-box protein, β-TRCP, which in turn recruits β-catenin to the SCF ubiquitin ligase complex so that it may be ubiquitinated and degraded [79]. When either APC is deleted or mutated, or β-catenin itself is mutated at its GSK-3β phosphorylation sites, either of which results in β-catenin accumulation and increased *Wnt* pathway signaling [80].

1.2.4. Von Hippel-Lindau Syndrome

Sensing of low oxygen by cells (hypoxia) prompts them to release signals, such as vascular endothelial growth factor (VEGF), that promote the growth of

blood vessels [81]. This signaling is mediated by the heterodimeric transcription factor, hypoxia-inducible factor (HIF). Under normoxic condition, N-terminal proline residues of one of the members of this heterodimer, HIFα, become hydroxylated [82]. Proline hydroxylation results in HIFα being recognized by an F-box protein, pVHL, and recruited to an SCF-like ubiquitin-ligase complex for proteasomal targeting [83]. Under hypoxic conditions, proline hydroxylation does not occur, thus allowing HIF to transcribe its target genes. pVHL is mutated in Von Hippel-Lindau syndrome (VHL), a cancer susceptibility syndrome characterized by the presence of hemangioblastomas, or blood vessel tumors of the CNS [84]. Thus, disease causing pVHL mutations prevent the recruitment and proteasomal degradation of HIFα, allowing for constitutive activation of HIF genes, which account for the hemangioblastoma phenotype due to constant VEGF production.

1.2.5. Cyclin E

hCdc4 is an F-box protein that targets cyclin E for degradation via the actions of the SCF ubiquitin ligase complex [85]. Cyclin E is expressed near the G0/G1 to S-phase transition, functioning as an activator of cyclin-dependent kinase 2 (Cdk2), which in turn facilitates the phase transition [86]. Therefore targeted degradation of cyclin E by the SCF^{hCdc4} complex is essential for preventing the aberrant transition to the S-phase in times of cellular crisis.

The elevation of cyclin E protein levels has been observed in a number of cancers, and appears due, in part, to the dysregulation of its degradation after the

transition to S-phase [87]. Mutations in hCdc4 have recently been identified in endometrial cancers as well as in a breast cancer cell line, both of which have dysregulated cyclin E. Underscoring the importance of this discovery, the hCdc4 gene resides in a region of a chromosome (4q32) that is deleted in over 30% of all human cancers [85,88].

1.3. Hypotheses for Proteasome Inhibitors' Therapeutic Index

It may not be readily apparent why the ubiquitin-proteasome pathway is a reasonable target for chemotherapeutic intervention. After all, every single cell has, and indeed relies heavily on, this pathway for cellular homeostasis. Below, we discuss several hypotheses as to why the proteasome pathway is a legitimate drug target for chemotherapy that will offer an acceptable therapeutic index.

1.3.1. Up-regulated Proteasome Components in Cancer

The proteasome pathway is hyperactivated in renal cell carcinomas, as compared to adjacent normal tissue, suggesting overall increases in protein degradation and increased cellular metabolism [89]. Furthermore, other groups have demonstrated increases in the expression of ubiquitin fusion proteins [90,91]. Just whether and how these alterations in proteasome function affect the pathogenicity of a particular cancer is an area of active investigation. However, even if the up-regulation of the proteasome pathway is simply an effect of cellular transformation, it suggests that, at least in some cases, the increase in activity plays an important role in the viability of these cancers.

1.3.2. Inhibition of Specific Proteasomal Substrate Degradation

As detailed above, several cancers have been linked to the aberrant degradation of particular proteasomal substrates. For example, the cyclin dependent kinase inhibitor, $p27^{kip1}$, has been studied extensively and is found to be down-regulated in many cancers, including breast, colon, and prostate [92,93]. If altered degradation of any substrates like $p27^{kip1}$ leads to oncogenic transformation, then inhibition of the proteasome pathway in these cases may have significantly greater effects on the cancer cells versus normal cells.

1.3.3. Converging Pathways

Maintaining control of cell growth is extremely important to the survival of multicellular organisms. Therefore, it is understandable that redundancy exists in the function of the molecules that control cellular proliferation, tumor suppressors. It is not surprising then, that the preponderance of evidence suggests that multiple genetic alterations in tumor suppressor pathways are necessary for oncogenic transformation [94]. As tumor cells undergo clonal expansion, they become increasingly genetically unstable, allowing for the accumulation of more mutations, in effect creating a population of cells related to, but characteristically different from, the progenitor cell [95,96]. This often leads to sub-populations of cells that can become resistant to chemotherapeutic regimens. One hypothesis explaining why proteasome inhibitors might be clinically successful is that, because of the critical role of the proteasome pathway in virtually all cellular

processes, few genetically variant cells can escape death, increasing the likelihood that these agents will be equally effective against all subpopulations.

1.3.4. Greater Sensitivity of Proliferating Cells to Proteasome Inhibitors

Often, chemotherapeutic strategies (e.g., DNA damaging agents) are said to have acceptable therapeutic indices due to the fact that cancer cells are rapidly proliferating, encountering cell-cycle checkpoints more frequently, and therefore need the inhibited processes more than nonproliferating cells. This theory is certainly reinforced when one considers the side-effects of such chemotherapeutic regimens: toxicity to rapidly proliferating cells such as gastrointestinal cells, hair follicles, etc.

In support of utilizing this hypothesis for proteasome inhibitors, several groups have demonstrated that proliferating cells undergo apoptosis when treated with proteasome inhibitors [97]. Proteasome inhibitors do not appear to be as effective at inducing apoptosis in quiescent cells [98]. Furthermore, the use of proteasome inhibitors in *in vivo* models has demonstrated significant reductions in tumor mass while remaining well tolerated by the model organisms, again suggesting sparing of quiescent cells [99].

A paradox is encountered, however, when one considers that proteasome inhibitors are effective against B-cell lymphomas, characteristically slow growing cancer cells [100]. Therefore, another characteristic of cancer cells that may be playing an important role in cellular sensitivity to chemotherapeutic agents, like proteasome inhibitors, is cellular stability. Cancer cells may be inherently

unstable due to the conditions leading to their oncogenic transformation, and that perturbation in global processes, such as DNA synthesis, push the balance towards apoptosis.

1.4. Proteasome Inhibitors as Antineoplastic Agents

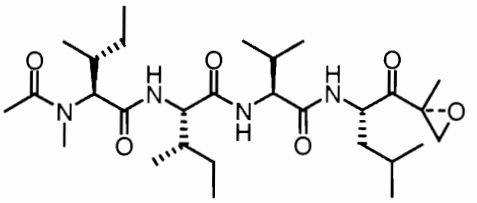
1.4.1. Proteasome Inhibitor Classes

Prior to the work presented in this thesis, there was only one established mechanism of action by which the proteasome pathway could be inhibited in intact cells: inactivation of the 26S proteasome's catalytic activity. There are three classes of proteasome inhibitors most often used to study the proteasome pathway: natural products, peptide aldehydes, and peptide-mimic boronic acids (Figure 1.5).

The natural product class consists of lactacystin and epoxomicin, and function by covalent reaction with the proteasome β-subunits' catalytic threonines [101,102]. Lactacystin is an inactive *Streptomyces* metabolite that is converted to the active metabolite, *clasto*-lactacystin β-lactone, by spontaneous transesterification. The catalytic threonines of the proteasome react with the β-lactone functional group irreversibly [101]. Epoxomicin is an *Actinomyces* metabolite that reacts with the proteasome via its epoxide functional group [102]. These inhibitors are less potent, but are more specific, than the peptide aldehydes.

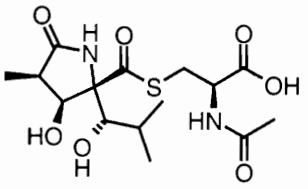
The peptide aldehydes work via a similar mechanism; they are peptide substrates containing electrophilic functional groups at their carboxyl-termini that react reversibly with the proteasome β-subunit catalytic threonines [103]. These

Natural Products

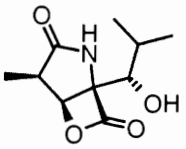


Epoxomicin

A.

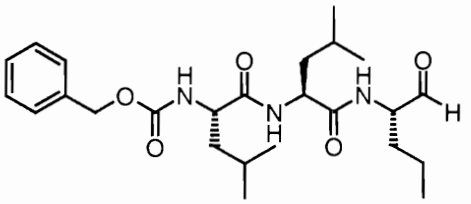


B.



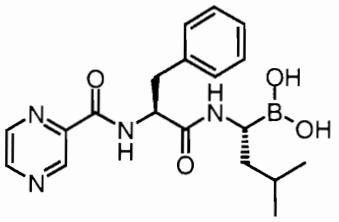
A. Lactacystin
B. *clasto*-Lactacystin β-lactone

Peptide Aldehydes



MG115

Peptide mimic boronic acids



VELCADE™ (PS-341)

Figure 1.5. Proteasome Inhibitor Classes. Shown are examples of natural product, peptide aldehyde, and peptide-mimic boronic acid proteasome inhibitors. These compounds all inhibit the catalytic sites of the proteasome. Prior to the work presented here, the only proteasome pathway inhibitors known were of these classes.

compounds are more stable and potent than lactacystin, but they tend to be less selective. The peptide aldehydes are also more readily accessible than lactacystin, due to their relatively low synthetic cost.

The third class of proteasome inhibitors is the peptide-mimic boronic acids [103]. These transition-state analogs are both potent and selective, forming reversible adducts with the proteasome β-subunit catalytic threonines. Of all of the proteasome inhibitors, only PS-341 is being pursued in cancer clinical trials.

1.4.2. Preclinical and Clinical Evaluation of the Proteasome Inhibitor, PS-341 (VELCADE™)

1.4.2.1. *In Vitro* Models of Human Disease

Given the prominent role of the ubiquitin-proteasome pathway in cellular homeostasis, it is not surprising that disruption of this pathway with proteasome inhibitors is toxic to cells. Indeed, many studies have proven this to be true, prompting the discovery of novel, potent, and selective proteasome inhibitors. Such a compound, VELCADE™ (PS-341), was discovered in a small molecule proteasome inhibitor library by ProScript, Inc. (now Millenium Pharmaceuticals, Inc.). PS-341 was chosen for development because it was found to be one of the most potent compounds from this library, in terms of causing cancer cell death and proteasome inhibition, and a high correlation exists between its cytotoxicity and proteasome inhibition (suggesting proteasome inhibition to be the primary mechanism of action) [104]. Like peptide based inhibitors, PS-341 is active against a wide range of human cancer cell lines (e.g., colon, pancreatic, prostate, etc.) [105].

Because of the unique mechanism of action of proteasome inhibitors, another trait of PS-341 is that it retains activity against cell lines that are resistant to other chemotherapeutic agents [100]. Furthermore, likely due to the role of the proteasome pathway in DNA repair, PS-341 has synergistic effects with a variety of DNA damaging agents (e.g., doxorubicin, CPT-11, and cisplatin) [106,107]. Combined, these favorable properties prompted the continued examination of this compound in animal models.

1.4.2.2. Animal Models of Human Disease

As with model cell lines, in *in vivo* models, PS-341 has demonstrated a favorable profile. Most importantly, in mouse xenografts, this compound was found to reach its intended site of action and inhibit the catalytic activity of the proteasome in the implanted tumor [99,104]. Furthermore, PS-341 appeared to be well tolerated at concentrations that inhibit the proteasome substantially. As in cell studies, PS-341 is active *in vivo* against a wide range of human cancers (e.g., multiple myeloma, colon, pancreatic, and prostate) [99,108]. It retains activity against cell lines that are resistant to other chemotherapeutic agents, has synergistic effects with a variety of DNA damaging agents (e.g., 5-fluorouracil, cisplatin, Taxol and adriamycin), and demonstrates selective toxicity toward malignant cells [109]. The positive findings of these studies prompted the continued examination of this compound in human clinical trials.

1.4.2.3. Human Clinical Trials

PS-341 has undergone Phase I clinical trials for solid tumors (43 patients, 14 different diagnoses) and hematologic malignancies (27 patients, 8 diagnoses) [110,111]. As in animal models, PS-341 was well tolerated at concentrations in which it inhibited the proteasome. In the solid tumor trial, the major dose-limiting complications were diarrhea and sensory neuropathy (with two of the highest dose patients experiencing grade 3 of each). In the hematologic malignancy trial, the major dose-limiting complications were thrombocytopenia and anemia (with 10 patients and 5 patients experiencing grade 3 or greater, respectively) [111]. Of course, when interpreting these dose-limiting toxicity data, one must consider that some of the complications encountered could be a reflection of treatments given prior to enrollment in these trials.

As in the animal models, tolerated doses were sufficient to significantly inhibit the proteasome in tumor samples (approximately 65 percent proteasome inhibition for both trials), demonstrating that PS-341 was reaching its intended target *in vivo*. This activity slowly diminished to approximately 20 percent inhibition 3 hours after dosing (one bolus).

Similar to what was observed *in vitro* and in animal models, PS-341 appears to affect hematological malignancies to a greater extent than solid tumors. In the solid tumor trial, only 1 patient out of 43 showed a partial response [111]. However, in the hematological malignancy trial the results were more promising [110]. Of the 9 multiple myeloma patients that were available for follow-up, 5 patients had a partial response, 3 patients showed disease stabilization, and one

patient has shown a full response, with no disease progression after 1 year. These results are remarkable when one considers that the patients in this trial had failed an average of four other standard therapies. This may be a reflection of what was observed in animal models; PS-341 can overcome cell resistance to standard therapies.

Another disease category that shows promise for successful PS-341 treatment is non-Hodgkin's lymphoma [110]. Two of the 10 non-Hodgkin's lymphoma patients showed partial responses. One of these patients had failed prior treatment with cyclophosphamide, doxorubicin, vincristine, and prednisone; autologous stem-cell transplantation; and radiation therapy. The second patient, who has a stable partial response after 12 months off therapy, failed prior treatment with cyclophosphamide, vincristine, prednisone, and rituximab.

When evaluating these data, it must be taken into consideration that the two trials were designed slightly differently; the major difference being that in the solid tumor trial, one cycle represented PS-341 for 2 weeks followed by 1 week of no treatment, while in the hematological malignancy trial, one cycle represented PS-341 for 4 weeks followed by 2 weeks of no treatment. Therefore, a different treatment regimen may be more effective for solid tumors. Also phase II trials are being planned for PS-341 in combination therapies in the hope that this approach will be more amenable to treating solid tumors.

Due to the promising results of the phase I trials, a phase II trial was initiated for approximately 200 multiple myeloma patients. This trial has recently been completed, however, the data for this study are not yet available. These

results were encouraging enough to enter a phase III multiple myeloma trial. This trial is currently underway with approximately 600 patients in 65 centers.

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CHAPTER 2

CYCLOPENTENONE PROSTAGLANDINS OF THE J SERIES INHIBIT THE UBIQUITIN ISOPEPTIDASE ACTIVITY OF THE PROTEASOME PATHWAY

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Cyclopentenone Prostaglandins of the J Series Inhibit the Ubiquitin Isopeptidase Activity of the Proteasome Pathway*

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Electrophilic eicosanoids of the J series, with their distinctive cross-conjugated α,β-unsaturated ketone, inactivate genetically wild type tumor suppressor p53 in a manner analogous to prostaglandins of the A series. Like the prostaglandins of the A series, prostaglandins of the J series have a structural determinant (endocyclic cyclopentenone) that confers the ability to impair the conformation, the phosphorylation, and the transcriptional activity of the p53 tumor suppressor with equivalent potency and efficacy. However, J series prostaglandins have a unique structural determinant (exocyclic α,β-unsaturated ketone) that confers unique efficacy as an apoptotic agonist. In seeking to understand how J series prostaglandins cause apoptosis despite their inactivation of p53, we discovered that they inhibit the ubiquitin isopeptidase activity of the proteasome pathway. In this regard, J series prostaglandins were more efficacious inhibitors than representative members of the A, B, or E series prostaglandins. Disruption of the proteasome pathway with proteasome inhibitors can cause apoptosis independently of p53. Therefore, this finding helps reconcile the p53 transcriptional independence of apoptosis caused by Δ12-prostaglandin J_2. This discovery represents a novel mechanism for proteasome pathway inhibition in intact cells. Furthermore, it identifies isopeptidases as novel targets for the development of antineoplastic agents.

Certain electrophilic prostaglandins (PG)[1] can repress transactivation by NFκB and p53 (1–4), two prominent transcription factors that govern the decision of a cell to survive or die (5–7). This transcriptional repression is a pharmacologically unique trait that distinguishes PG of the A and J series from other PG that act via membrane-spanning receptors (8). If the endocyclic α,β-unsaturated ketone shared by A and J series PG confers activity, as hypothesized (1, 2, 4, 9, 10), then two predictions should be valid. First, individual A series and J series PG should act rather uniformly on the cellular processes they affect. Second, their cellular effects should be self-consistent with established models of NFκB and p53 function. However, not all experiments affirm these predictions. For example, the A and J series PG both repress NFκB transcription and inhibit IκB kinase (1, 2); however, only Δ12-PGJ_2 is anti-inflammatory (11). Likewise, the A and J series PG both repress p53 transcription; however, only the A series PG antagonize p53-dependent apoptosis (Ref. 4 and see below).

Herein we report the discovery of a molecular mechanism that clarifies the distinctive cellular effects of cyclopentenone PG. Namely, J series PG preferentially inhibit the ubiquitin isopeptidase activity (ubiquitin-specific protease) of the proteasome pathway. This pathway is the major nonlysosomal degradation pathway in cells (12, 13). The degradation of target proteins via this pathway largely depends on their covalent modification with a ubiquitin polymer. This polymer consists of ubiquitin (8.5 kDa) subunits that are covalently attached via isopeptide bonds between the COOH terminus of one ubiquitin and the lysine ε-amino group of another ubiquitin. In turn, this polymer is covalently attached to a lysine ε-amino group on the target protein via another isopeptide bond. Ubiquitin isopeptidases are a large family of cysteine hydrolases that preferentially cleave these isopeptide bonds (14).

We demonstrate that inhibition of ubiquitin isopeptidase activity by PG depends on nuances of olefin-ketone conjugation. J series PG with a cross-conjugated α,β-unsaturated dienone are more efficacious inhibitors compared with A series PG with a single α,β-unsaturated ketone (Fig. 1). We further demonstrate that J series PG cause apoptosis, concurrently with inhibition of isopeptidase activity, and independently of p53-mediated gene transactivation. Our discovery has particular implications for the development of novel antineoplastic agents that are effective against cancers with dysfunctional p53 pathways.

EXPERIMENTAL PROCEDURES

Materials—We used Dulbecco's minimum essential medium and media supplements (Life Technologies, Inc.); Δ12-PGJ_2, PGA_1, PGA_2, PGB_1, PGE_2, and 15-keto-PGE_2 (Cayman Chemicals, Ann Arbor, MI); etoposide (Sigma); complete protease inhibitor mixture and FuGene-6 transfection reagent (Roche Molecular Biochemicals); enhanced chemiluminescence reagents (Amersham Pharmacia); luciferase reporter lysis buffer and reporter detection reagents (Promega); antibodies directed against p53, PAb240, FL393, and DO-1 (Santa Cruz Biotechnology), Phospho-p53 (Ser^{15}) (Cell Signaling Technology), and PAb1620 (Oncogene Research Products); antibodies against $p21^{WAF1/CIP1}$ (C-19, Santa Cruz Biotechnology) and ubiquitin (Ubi-1, Zymed Laboratories Inc.); horseradish peroxidase-conjugated secondary antibodies; protein A/G PLUS-Agarose (Santa Cruz Biotechnology); z-LLE-MCA, z-LLL-MCA, MG115, Ac-DEVD-MCA (Peptides International); LLVY-MCA, z-LRGG-MCA (Alexis Corporation); MDL 28170 (z-VF-CHO, Calbiochem); Ub-^{125}I-lysozyme (gift from Dr. Martin Rechsteiner, Department of Biochemistry, University of Utah); annexin V, Alexa Fluor® 488 conjugate (Molecular Probes, Inc.); a luciferase p53 cis reporter con-

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[1] The abbreviations used are: PG, prostaglandin(s); Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-methylcoumarin-7-amide; IκB, inhibitor of NFκB; NFκB, nuclear factor κB; z-LLE-MCA, carbobenzoxy-L-leucyl-L-leucyl-L-glutamate α-(4-methyl-coumaryl-7-amide); z-LLL-MCA, carbobenzoxy-L-leucyl-L-leucyl-L-leucine α-(4-methyl-coumaryl-7-amide); LLVY-MCA, succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine α-(4-methyl-coumaryl-7-amide); z-LRGG-MCA, carbobenzoxy-L-leucyl-L-arginyl-L-glycyl-L-glycine α-(4-methyl-coumaryl-7-amide); MG115, carbobenzyloxy-L-leucyl-L-leucyl-norvaline; MDL 28170, carbobenzoxy-valinyl-phenylalaninal; 4-HNE, 4-hydroxynonenal; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ANOVA, analysis of variance.

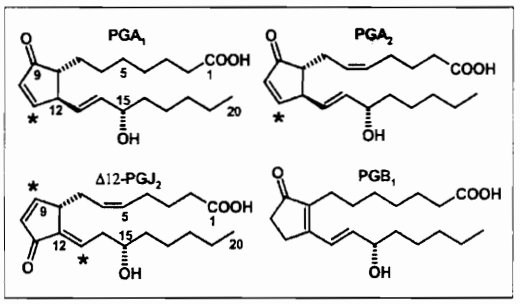

FIG. 1. **Structures of cyclopentenone prostaglandins.** *Asterisks* depict electrophilic carbon(s). PGB_1 is relatively inert in physiological milieu.

struct (p53-Luc, Stratagene); β-galactosidase expression vector (pSV-β; Promega); and pC53-SN3 (wild type) and pC53-SCX3 (V143A mutant) p53 constructs ((15, 16), gift from Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD).

Cell Culture—We maintained RKO, RKO-E6 (gift from Dr. Mark Meuth, Institute for Cancer Studies, University of Sheffield, Sheffield, United Kingdom), Ts20 cells (17) (gift from Dr. Harvey Ozer, Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, Newark, NJ) in Dulbecco's minimum essential medium in a humidified incubator with 5% CO_2. We supplemented the media with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin, and streptomycin, and 10% (v/v) fetal bovine serum. We maintained Ts20 cells at 32 °C (permissive temperature) but placed them in a 40 °C incubator (nonpermissive temperature) to inactivate the E1 ubiquitin-activating enzyme in experiments designed to observe disassembly of polyubiquitinated proteins (17). All other cells were maintained at 37 °C.

p53-DNA Binding and Transactivation—We measured p53-DNA binding activity via electrophoretic mobility shift assay. We isolated nuclei and generated protein-DNA complexes as described previously (18). We used the wild type p53 consensus, 5′-AGC TGG ACA TGC CCG GGC ATG TCC-3′, double-stranded oligonucleotide, and a mutant oligonucleotide, 5′-AGC TGG ATC GCC CCG GGC ATG TCC-3′ (Geneka), to evaluate sequence specific p53-DNA binding. We incubated 25,000 cpm (2×10^7 cpm/μg) of the ^{32}P-labeled oligonucleotide probe with 1 μg of nuclear extract. We fractionated samples on a nondenaturing 6% TBE (45 mM Tris borate, 1 mM EDTA)-polyacrylamide gel. We exposed Kodak XAR film to the gel for 8–24 h at −70 °C to detect p53-DNA complexes.

We transfected RKO cells with a p53-luciferase reporter construct to measure p53-dependent transactivation (4). We used expression of an independently transfected β-galactosidase gene to normalize for transfection efficiency and cell death.

Immunochemical Detection of Proteins—After treating cells with the indicated compounds for the indicated times, we removed the medium and lysed cells in 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM EDTA with 0.1% SDS, 0.1% deoxycholate, 1× complete protease inhibitor mixture. We measured protein concentration by the Bradford method. We fractionated equal portions of the total cell lysate from each sample (12.5 μg of protein) by SDS-PAGE. We transferred proteins to polyvinylidene difluoride blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline T (20 mM Tris·HCl, pH 7.5, 100 mM sodium chloride, 0.5% (v/v) Tween 20). We measured proteins immunochemically by using primary antibodies directed against p53 (1:4,000), $p21^{WAF1/CIP1}$ (1:5,000), and ubiquitin (1:1000), followed by horseradish peroxidase-conjugated secondary antibodies (1:4000). We detected antigen-antibody complexes with enhanced chemiluminescence reagents. We scanned gels and quantified intensities using Kodak 1D Image Analysis Software.

Immunoprecipitation of p53—We treated RKO and RKO-E6 cells with the indicated compounds for the indicated times. We lysed cells in 250 mM sucrose, 50 mM Tris, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1× complete protease inhibitor, 1 mM NaF, and 1 mM sodium orthovanadate. We sonicated the lysate twice for 5 s at 4 °C. After centrifugation at 13,000 × *g*, we incubated samples containing 200 μg of total protein, in 1 ml of lysis buffer, with 1 μg of primary antibody, and 20 μl of protein A/G PLUS-Agarose overnight at 4 °C on a rocker. PAb 1620 precipitates p53 occurring in a wild type conformation; PAb 240 precipitates p53 in a mutant conformation (19–21). DO-1 precipitates p53 occurring in either conformation or as a p53-ubiquitin conjugate. We centrifuged the samples at 2500 × *g* to isolate the antigen-antibody-bead complexes. We washed the immunoprecipitate four times with 1 ml of phosphate-buffered saline, 0.4% Tween 20. We then measured p53 or ubiquitin by immunochemical analysis with antibodies for p53 (FL-393) and ubiquitin (Ubi-1), respectively. Note that PAb 240 and PAb 1620 are monoclonal antibodies, despite their annotation.

Apoptosis Assays—We incubated 5×10^4 RKO cells, plated on a Lab-Tek® II Chamber Slide™ (Nalge Nunc International Corp.), with the indicated compounds for the indicated times and then washed the cells twice with phosphate-buffered saline. We added 100 μl of annexin-binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) and 5 μl of Alexa Fluor® 488-labeled annexin V and incubated samples for 15 min at room temperature. We washed the cells twice with 400 μl of annexin-binding buffer and resuspended them in 15 μl of 50:50 annexin-binding buffer:water. We stained the cells with propidium iodide (1 μM) and Hoechst 33342 (1 μM) and examined them by fluorescence microscopy. We determined caspase activity and DNA fragmentation as described previously (22).

$p21^{WAF1/CIP1}$ Gene Expression—We determined $p21^{WAF1/CIP1}$ mRNA expression from cDNA microarray experiments (4).

20 S Proteasome Activity Assays—We determined 20 S proteasome catalytic activity by monitoring the cleavage of substrates for the post-glutamyl peptidase (z-LLE-MCA) and chymotrypsin-like (z-LLL-MCA and LLVY-MCA) proteolytic activities of the 20 S proteasome. Briefly, we incubated RKO cells with the indicated compounds for the indicated times. We lysed cells in 250 μl of 25 mM HEPES, 5 mM EDTA, 0.1% CHAPS, 5 mM ATP, pH 7.5 with 2 mM dithiothreitol added just prior to lysis. We sonicated lysates and dispensed 45-μl aliquots into four separate wells of a 96-well plate. We added 5 μl of each proteasome substrate (500 μM in lysis buffer) to separate 45-μl samples (final substrate concentration: 50 μM). We incubated the assay mixtures at 37 °C for 15 min and quantified fluorescence (substrate cleavage) by exciting the samples at λ = 360 nm and monitoring the emission at λ = 460 nm.

26 S Proteasome Activity Assay—We determined the 26 S proteasome regulatory activity by monitoring the cleavage of Ub-^{125}I-lysozyme, prepared as described previously (23). We incubated RKO cells with the indicated compounds for the indicated times. To monitor 26 S proteasome regulatory activity, we incubated 22.5 μl of lysate, 2.5 μl of an ATP regenerating system (0.5 mM ATP, 60 μg/ml creatine phosphokinase, 6.6 mM phosphocreatine, 10 mM Tris-HCl, 0.5 mM $MgCl_2$, 1 mM KCl, 0.05 mM dithiothreitol), and 25 μl of ^{125}I-lysozyme-ubiquitin conjugates (136 cpm/μl). After 30 min at 37 °C, we removed a 20-μl sample and added 400 μl of bovine serum albumin solution (1% bovine serum albumin in 10 mM Tris, pH 7.5, 0.02% azide). We then isolated the acid soluble fraction by trichloroacetic acid precipitation (50 μl 100% w/v trichloroacetic acid, on ice, 15 min) and centrifugation for 15 min at 16,000 × *g*. We determined the amount of ^{125}I in the supernatant by γ spectrometry and calculated the percentage of soluble ^{125}I (supernatant ^{125}I/total ^{125}I) to determine the extent of substrate proteolysis.

Isopeptidase Activity Assay—We determined isopeptidase activity by monitoring cleavage of a peptide substrate (z-LRGG-MCA) that mimics the carboxyl terminus of ubiquitin, which is involved in isopeptide bond formation. We observed that this substrate was subject to degradation by the proteasome; therefore, we incubated RKO cell lysates (0.5 μg/ml in 25 mM HEPES, 5 mM EDTA, 0.1% CHAPS, 5 mM ATP, pH 7.5) with 30 μM MG115 to inhibit proteasome activity (>90% inhibition was achieved). These conditions were used to eliminate background proteolytic activity and to permit selective determination of isopeptidase activity. We next added various concentrations of prostaglandins to identify isopeptidase inhibitory activity. Prostaglandins were incubated for 0.5 h on ice prior to substrate addition. We incubated the assay mixtures at 37 °C for 3 h and quantified fluorescence as described above. Lysates were also monitored for proteasome activity to ensure that the isopeptidase inhibitory effect seen with the prostaglandins was not due to additive proteasome inhibition.

RESULTS

Δ12-PGJ_2 Impairs the p53 Tumor Suppressor while Inducing Apoptosis Concurrently—PGA_1 and A_2 can repress transcription by p53, thereby antagonizing p53-dependent apoptosis (4). If their electrophilic cyclopentenone ring confers this activity, as we hypothesized, then Δ12-PGJ_2 should act analogously to PGA_1 and PGA_2. Consistent with our hypothesis, we observed that RKO cells exposed to Δ12-PGJ_2 acquired p53 that was

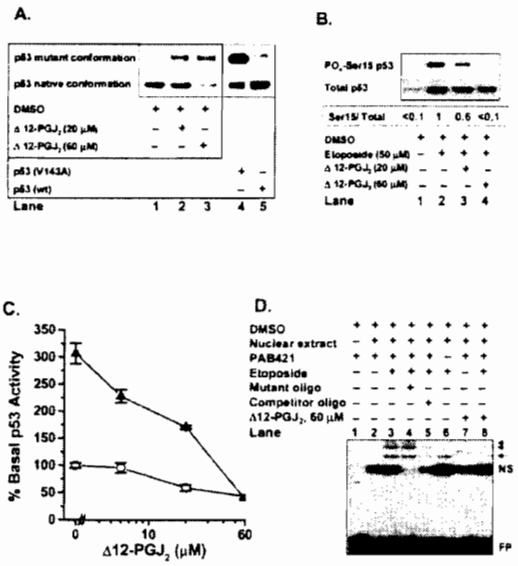

FIG. 2. **Δ12-PGJ$_2$ impairs p53 conformation and transactivation.** In *panels A–C*, RKO cells were incubated with Δ12-PGJ$_2$ (0–60 μM, 6 h, 37 °C). *A*, Δ12-PGJ$_2$ causes p53 to obtain a mutant conformation. The p53 protein in cell lysates was immunoprecipitated with conformationally sensitive antibodies that distinguish between the native (Pab1620) and mutant (Pab240) conformers of p53. Positive controls were RKO cells transfected with plasmids that express either a conformational mutant of p53 (*p53 (V143A)*) or wild type p53 (*p53 (wt)*). *B*, Δ12-PGJ$_2$ inhibits a key step in p53 activation, serine 15 phosphorylation. Total p53 protein and p53 phosphorylated at serine 15 (*PO$_4$-Ser15 p53*) were determined by Western blot analysis. Densitometry was used to obtain the PO$_4$-Ser^{15} p53/total p53 ratios. This ratio in *lane 2* (the positive control, etoposide) is designated as 1 for comparison purposes. *C*, Δ12-PGJ$_2$ inhibits the transcriptional activity of p53. Δ12-PGJ$_2$ inhibited the basal transactivation (○) and etoposide-induced transactivation (▲) of a p53-luciferase reporter plasmid in RKO cells. Data are the mean ± S.D., n = 3 experiments. *D*, Δ12-PGJ$_2$ inhibits DNA binding by p53. RKO cells were incubated for 6 h with Me$_2$SO vehicle, 50 μM etoposide, 60 μM Δ12-PGJ$_2$, alone, or combined with 50 μM etoposide. Nuclear extracts from these cells were incubated with ^{32}P-labeled oligonucleotide containing a consensus binding site for p53, and as indicated, with PAB 421, an antibody that binds p53, excess cold oligonucleotide (competitor) identical to the ^{32}P-labeled oligonucleotide, or excess cold oligonucleotide (mutant) differing in sequence from ^{32}P-labeled oligonucleotide. *Arrows* indicate p53-^{32}P-labeled oligonucleotide complexes. *NS* = nonspecific labeling; *FP* = free ^{32}P-labeled oligonucleotide.

transcriptionally inactive, as determined by a variety of assays. Δ12-PGJ$_2$ transforms p53 protein in RKO cells from its wild type, native conformation into a mutant conformation (Fig. 2A, *lanes 1–3*). This mutant p53 conformation is a less efficient substrate for serine 15 phosphorylation. Phosphorylation of this residue on p53 is an early event in p53 activation. Etoposide increased total and phosphoserine 15 p53 in RKO cells relative to the control (Fig. 2B, *lanes 1* and *2*). However, coincubation with 20–60 μM Δ12-PGJ$_2$ reduced serine 15 phosphorylation (Fig. 2*B*, *lanes 3* and *4*). The consequences of inhibiting this activating step are reflected in the transactivating ability of p53. Etoposide-stimulated transcription of a p53-dependent reporter gene was potently inhibited by 6–60 μM Δ12-PGJ$_2$ (Fig. 2*C*). It became apparent why p53 failed to transcribe genes when we examined its ability to bind an oligonucleotide containing a p53 consensus binding sequence. In this assay, etoposide (50 μM) promoted the formation of p53-^{32}P-labeled oligonucleotide complexes (Fig. 2*D*, *lane 6*) that could be supershifted with anti-p53 antibody (Fig. 2*D*, *lane 3*), and competitively inhibited by excess "cold" oligonucleotide (Fig. 2*D*, *lane 5*). Excess cold mutant oligonucleotide inhibited only the nonspecific formation of p53-^{32}P-labeled oligonucleotide complexes (Fig. 2*D*, *lane 4*). However, 60 μM Δ12-PGJ$_2$, alone (Fig. 2*D*, *lane 7*) or combined with etoposide (Fig. 2*D*, *lane 8*) inhibited the formation of p53-^{32}P-labeled oligonucleotide complexes. These data suggest that, in terms of their pharmacological effects on p53, J series PG are quantitatively and qualitatively indistinguishable from A series PG. Cyclopentenone PG have similar effects on wild type p53 in cell lines other than RKO cells, including HCT 116, A549, and MCF-7 cells (data not shown).

In contrast to their uniform effects on p53 function, A and J series PG had divergent effects on apoptosis. As expected for agents that impair p53 function, PGA$_1$ and PGA$_2$ do not cause apoptosis (Fig. 3). However, Δ12 PGJ$_2$ causes apoptosis, characterized by nucleosomal fragmentation, activation of caspase-3, and cellular association of annexin V (Fig. 3, *A—C*). The induction of apoptosis by Δ12-PGJ$_2$, concurrent with its impairment of p53 function in RKO cells is difficult to reconcile with the conventional model of p53 tumor suppression (6, 7).

p21$^{WAF1/CIP1}$ Protein Levels Rise, Despite the Decline in Its Transcription—As expected, transcription of endogenous genes regulated by p53, typified by p21$^{WAF1/CIP1}$, declined in RKO cells incubated with Δ12-PGJ$_2$ (Fig. 4*A*). However, we noticed that these cells continued to accumulate p21$^{WAF1/CIP1}$ protein (Fig. 4*B*, *lane 4*). This implies that Δ12-PGJ$_2$ had slowed the rate of p21$^{WAF1/CIP1}$ protein degradation. This effect is distinctive for Δ12-PGJ$_2$; mRNA and protein levels of p21$^{WAF1/CIP1}$ fall, in parallel, in RKO cells treated with PGA$_1$ or PGA$_2$ (4) (Fig. 4*A* and 4*B*, *lanes 2* and *3*). Proteolysis via the ubiquitin-dependent proteasome pathway modulates the activity of several transcription factors, tumor suppressors, and regulatory proteins, including p21$^{WAF1/CIP1}$ and p53 (24, 25). Inhibitors of the 20 S proteasome catalytic activity can also cause apoptosis (26). Therefore, we hypothesized that Δ12-PGJ$_2$ inhibited the ubiquitin-dependent 26 S proteasome pathway.

We used RKO and RKO-E6 cells as a model to test this hypothesis. RKO-E6 cells are derived from isogenic RKO cells stably transfected with the E6 oncoprotein. The E6 oncoprotein is an ubiquitin-conjugating enzyme that hastens proteasome-mediated degradation of the p53 tumor suppressor protein (27). One predicts that RKO-E6 cells will accumulate p53 to a lesser extent than will RKO cells under various conditions ranging from basal to genomic stress. In contrast, one predicts that both cell lines will accumulate p53 to a similar extent if Δ12-PGJ$_2$ inhibits the ubiquitin-dependent proteasome pathway.

Δ12-PGJ$_2$ Inhibits the Ubiquitin-dependent Proteasome Pathway—Consistent with our prediction, the ratio of p53 protein in RKO-E6 cells/RKO cells was lower in every case tested, except cells incubated with Δ12-PGJ$_2$ (Fig. 5*A*, *lane 5*). Note that RKO-E6 cells incubated with etoposide accumulate 3-fold less p53 compared with isogenic RKO cells (Fig. 5A, *lane 2*), confirming that the E6 oncoprotein hastens the degradation of p53. Note also that RKO-E6 cells incubated with PGA$_1$ and PGA$_2$ accumulated negligible amounts of p53 compared with the vehicle control (Fig. 5*A*, *lanes 3* and *4 versus lane 1*), indicating that these cyclopentenone PG do not inhibit proteasome-mediated degradation of p53 as efficaciously as Δ12-PGJ$_2$. Both RKO and RKO-E6 cells incubated with Δ12-PGJ$_2$ accumulated appreciable p53 protein (~2–3-fold compared with vehicle control (Fig. 5*A*, *lane 5 versus lane 1*). A separate control experiment showed that the 20 S proteasome inhibitor, MG115, affected RKO and RKO-E6 cells in a manner analogous to Δ12-PGJ$_2$ (Fig. 5*A*, *lane 6 versus lane 5*).

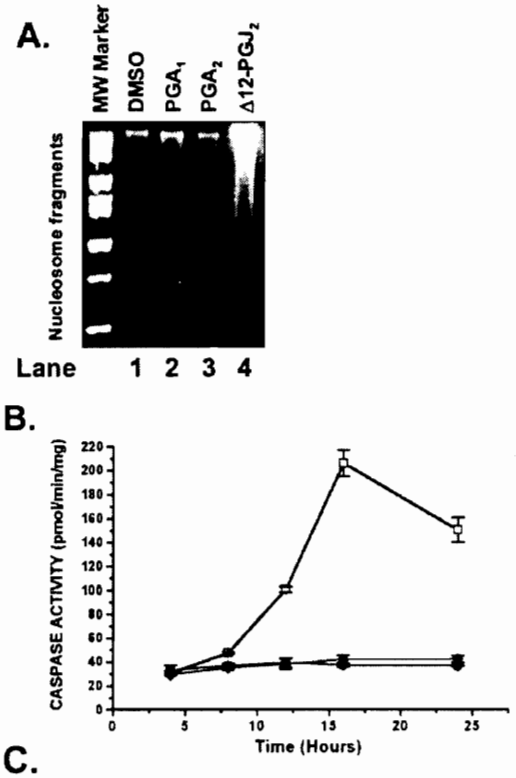

FIG. 3. **Δ12-PGJ$_2$ causes apoptosis despite inactivating p53-mediated transcription.** *A*, only Δ12-PGJ$_2$ causes nucleosomal DNA fragmentation. RKO cells were treated with vehicle or 20 μM Δ12-PGJ$_2$, PGA$_1$, or PGA$_2$ for 16 h. DNA was isolated from nuclear extracts, subjected to electrophoresis on a 1.5% agarose gel, and visualized by staining with ethidium bromide. *B*, only Δ12-PGJ$_2$ causes the activation of caspase-3. RKO cells were treated for 16 h with either vehicle (●), 20 μM Δ12-PGJ$_2$ (□), or PGA$_1$ (♦), and caspase-3 activity was determined. Data are the mean ± S.D., $n = 3$ experiments. *C*, only Δ12-PGJ$_2$ causes externalization of cellular phosphatidylserine. Externalization of phosphatidylserine is a marker for apoptosis. RKO cells were treated with vehicle (*panel i*), 20 μM PGA$_2$ (*panel ii*), 20 μM Δ12-PGJ$_2$ (*panel iii*), or 50 μM etoposide (*panel iv*) for 16 h. Fluorescence microscopy was used to determine the extent Alexa Fluor® 488-labeled annexin V (anti-phosphatidylserine) at their extracellular surface.

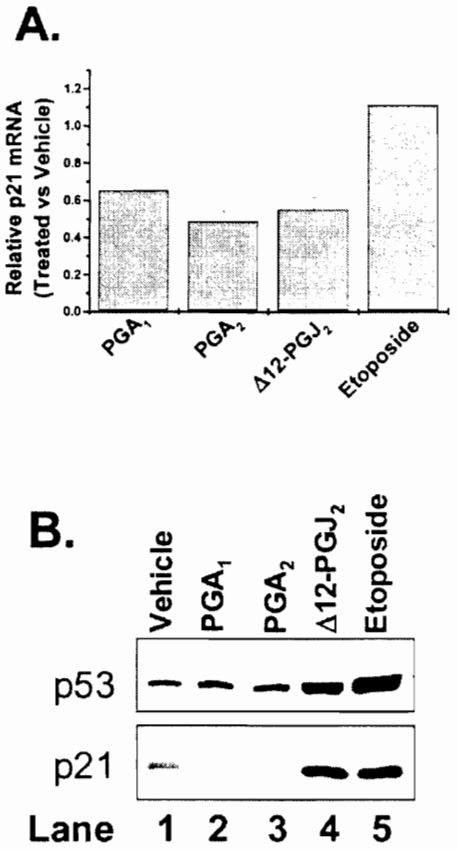

FIG. 4. **Δ12-PGJ$_2$ causes p21$^{WAF1/CIP1}$ protein accumulation despite antagonizing p21 transcription.** RKO cells were treated with etoposide (50 μM), PGA$_1$ (60 μM), PGA$_2$ (60 μM), or Δ12-PGJ$_2$ (60 μM), for 6 h. *A*, total RNA was isolated for microarray analysis to determine relative p21 mRNA expression. *B*, cellular levels of p53 and p21$^{WAF1/CIP1}$ protein were determined by immunochemical analysis.

Cellular p53 proteins are targeted for 26 S proteasome-mediated degradation by conjugation with polyubiquitin (28). Consistent with our hypothesis, Δ12-PGJ$_2$ caused RKO and RKO-E6 cells to accumulate high molecular weight p53 epitopes, recognized by anti-p53 antibody (Fig. 5*B*, *lanes 5* and 10). Immunoprecipitation with anti-p53 antibody, followed by SDS-PAGE and immunochemical analysis with anti-ubiquitin antibody, confirmed that these high molecular weight p53 epitopes were polyubiquitinated isoforms of p53 (Fig. 5*C*, *lanes 5* and 6). The accumulation of p53-polyubiquitin isoforms in Fig. 5*C* signifies that Δ12-PGJ$_2$ inhibits the ubiquitin-dependent proteasome pathway in a concentration-dependent manner. This effect is not restricted to p53 or to RKO cells; Δ12-PGJ$_2$ also caused a corresponding accumulation of polyubiquitin conjugates in HL-60 cells that lack p53 alleles (data not shown). Polyubiquitin conjugate accumulation was most pronounced in cells treated with J series PG (Fig. 5*D*, *lanes 2–4*) compared with PGA$_1$, PGA$_2$, PGB$_1$, 15-keto-PGE$_2$, or PGE$_2$ (Fig. 5*D*, *lanes 5–9*). J series PG share a cross-conjugated unsaturated ketone, while the other PG do not have this chemical feature. Note that while PGJ$_2$ does not possess cross-conjugation, it is rapidly converted to Δ12-PGJ$_2$ *in situ* (29).

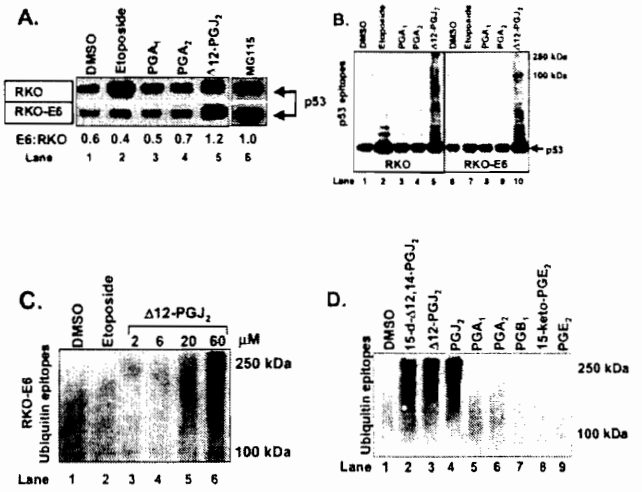

FIG. 5. **Δ12-PGJ$_2$ inhibits the ubiquitin-dependent proteasome pathway in RKO and RKO-E6 cells.** RKO and/or RKO-E6 cells were treated with the indicated PG (20 μM, 6 h) or etoposide (50 μM, 6 h). *A*, Δ12-PGJ$_2$ inhibits HPV-E6-mediated degradation of p53. Cellular levels of p53 protein were determined by immunochemical analysis. MG115 (20 μM, 4 h) was used as a positive control for inhibiting proteasome-mediated degradation of p53. Densitometric analysis was used to obtain relative p53 abundance ratios. *B*, Δ12-PGJ$_2$ causes the accumulation of high molecular weight p53. Prolonged exposure of the Western blot in *A* was used to identify high molecular weight epitopes recognized by anti-p53. *C*, Δ12-PGJ$_2$ causes the accumulation of polyubiquitinated p53. p53 was immunoprecipitated with anti-p53, fractionated by SDS-PAGE, and analyzed immunochemically with an antibody to ubiquitin. *D*, only J series PG cause the accumulation of polyubiquitin conjugates. Whole cell lysates were collected from RKO cells that were treated as indicated. Ubiquitin was then analyzed immunochemically.

It is unlikely that Δ12-PGJ$_2$ inactivated the E6 oncoprotein, or other endogenous ubiquitin ligases, ubiquitin-activating enzymes, or ubiquitin-conjugating enzymes, because cells incubated with Δ12-PGJ$_2$ accumulated p53-polyubiquitin conjugates. This implies that these enzymes are still functional in their roles of catalyzing ubiquitin conjugation (Fig. 5*B*). Δ12-PGJ$_2$ retained its potency and efficacy as an apoptotic agonist in RKO-E6 cells (data not shown). This is consistent with our conclusion that PGJ$_2$ causes apoptosis via a process that is independent of p53 transcription.

Δ12-PGJ$_2$ Inhibits the Proteasome Pathway at a Site Distinct from the 26 S Proteasome—The 26 S proteasome consists of multiple subunits, including two 19 S regulatory subunits that recognize and bind polyubiquitinated protein substrates, and a 20 S proteolytic subunit having broad substrate specificity (24). MG115, which acts at the 20 S catalytic site, inhibited hydrolysis of several proteasome substrates in whole cell lysates. We examined hydrolysis of substrates for the chymotrypsin-like (z-LLL-MCA and LLVY-MCA) and post-glutamyl peptidase (z-LLE-MCA) proteolytic activities, as well as a 19 S regulatory subunit-dependent substrate, Ub-^{125}I-lysozyme (Fig. 6*A*). At a concentration exceeding that which causes polyubiquitin conjugate accumulation in intact cells, Δ12-PGJ$_2$ (60 μM) did not significantly inhibit hydrolysis of any of these proteasome substrates (Fig. 6*A*). In other words, MG115 (20 μM) is significantly more efficacious ($\geq$90%) than Δ12-PGJ$_2$ ($\leq$20%) at inhibiting the catalytic activity of the 26 S proteasome. Lactacystin, another agent that acts at the 20 S catalytic site, behaved similarly (data not shown). We noted that the differences in efficacy between MG115 and Δ12-PGJ$_2$ as proteasome inhibitors (Fig. 6*A*) did not correlate with the extent or rate of polyubiquitin accumulation in cells. A dose-response experiment showed that, despite being a weaker proteasome inhibitor, Δ12-PGJ$_2$ (6–60 μM) (Fig. 6*B*, *lanes 5–7*) caused intracellular polyubiquitin accumulation to a greater extent than MG115 (2–20 μM) (Fig. 6*B*, *lanes 2–4*). Furthermore, we observed that the molecular weight distribution of polyubiquitin differed between RKO cells treated with MG115 and Δ12-PGJ$_2$. When incubated with 20 μM MG115, RKO cells accumulated polyubiquitin distributed around a maximum of ~160 kDa. When incubated with 60 μM Δ12-PGJ$_2$, RKO cells accumulated polyubiquitin distributed around a maximum of ~250 kDa (Fig. 6*C*). Collectively, these data (Figs. 5 and 6) suggest that Δ12-PGJ$_2$ inhibits the terminal step in the proteasome pathway: isopeptidase-mediated disassembly of polyubiquitin.

Δ12-PGJ$_2$ Inhibits the Isopeptidase Activity of the Proteasome Pathway—To test our hypothesis, we sought an experimental system that would allow us to examine polyubiquitin disassembly in the absence of *de novo* ubiquitin conjugation. The murine cell line, ts20, harbors a temperature-sensitive E1 ubiquitin-activating enzyme that is catalytically impaired at the nonpermissive temperature, 40 °C (17). We first uniformly accumulated polyubiquitinated proteins at the permissive temperature (32 °C) by incubating ts20 cells with MG115 (1 μM, 12 h). We then added fresh media containing either MG115 (6 μM) or Δ12-PGJ$_2$ (20 μM) and immediately incubated the samples at the nonpermissive temperature and monitored polyubiquitin disassembly by ubiquitin isopeptidases (Fig. 7*A*). Under these conditions, Δ12-PGJ$_2$ slowed the rate of polyubiquitin disassembly significantly (Fig. 7B, *lanes 8–10*) compared with Me$_2$SO (Fig. 7*B*, *lanes 2–4*) or MG115 (Fig. 7*B*, *lanes 5–7*). Densitometric analysis of the polyubiquitinated proteins in Fig. 7*A* showed that, in the absence of *de novo* ubiquitin conjugation, polyubiquitin disappeared by first-order kinetics with a half-life ($t_{1/2}$) $\cong$ 6 h in ts20 cells treated with 20 μM Δ12-PGJ$_2$ (Fig. 7*C*). This rate is 3-fold slower than the rate of polyubiquitin disappearance, $t_{1/2} \cong$ 2 h, in ts20 cells treated with MG115, consistent with Δ12-PGJ$_2$ and MG115 having different sites of action. We note that this effect could not be reproduced with a cell-penetrable, peptide inhibitor of cathepsin B and calpains (MDL 28170 (30)), indicating that polyubiquitin disassembly does not reflect activity of other major cellular proteolytic pathways (data not shown). We also note that ts20 cells respond to PG treatment in a manner similar to RKO cells; Δ12-PGJ$_2$ causes a greater accumulation of polyubiquitin conjugates in ts20 cells than an equimolar concentration of MG115 (data not shown).

Isopeptidases comprise a large family of cysteine proteases. Currently, little is known about the substrate specificity of the individual members of this family. Therefore, we measured inhibition of isopeptidase activity by quantifying the degradation of a general isopeptidase substrate, z-LRGG-MCA, that mimics the ubiquitin COOH-terminal isopeptide linkage (Fig. 8). We observed a concentration-dependent inhibition of isopeptidase substrate degradation with Δ12-PGJ$_2$, while PGA$_1$, PGB$_1$, PGE$_2$, and 15-keto-PGE$_2$ demonstrated no inhibitory

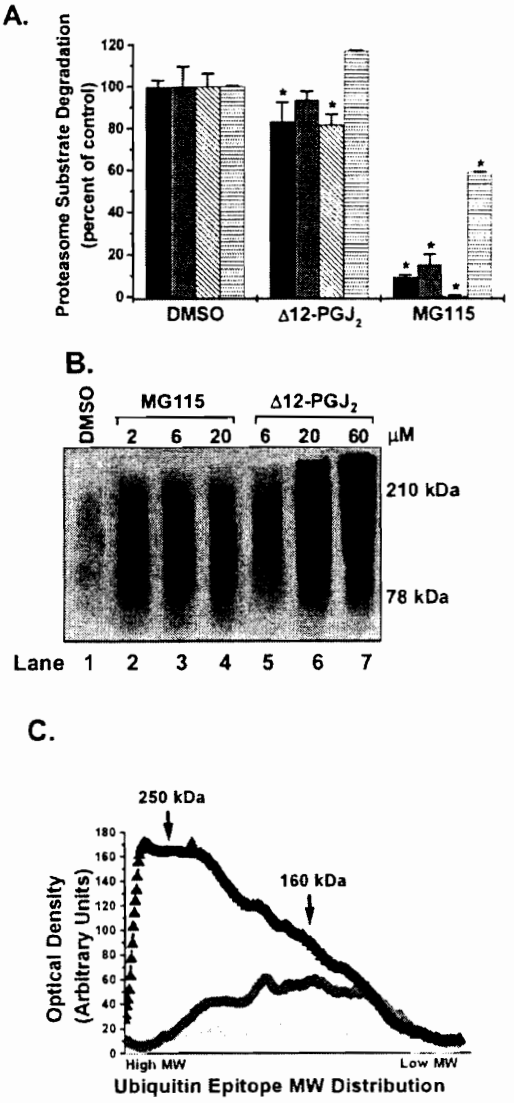

FIG. 6. **$\Delta 12\text{-PGJ}_2$ inhibits the ubiquitin-dependent proteasome pathway at a site distinct from the proteasome.** *A*, $\Delta 12\text{-PGJ}_2$ does not inhibit the catalytic or regulatory activities of the proteasome. RKO cells were incubated for 6 h at 37 °C with Me_2SO (vehicle), 20 μM MG115, or 60 μM $\Delta 12\text{-PGJ}_2$. Cell lysates were analyzed for proteolysis of catalytic subunit substrates (ZLLE-MCA (■), ZLLL-MCA (□), and LLVY-MCA (▨)) and a regulatory subunit-dependent substrate (Ub-^{125}I-lysozyme (■)). Data are the mean ± S.D., $n = 3$, with statistical significance indicated (*, ANOVA, $p < 0.05$). *B*, despite being a weak proteasome inhibitor, $\Delta 12\text{-PGJ}_2$ is potent at causing polyubiquitin accumulation. RKO cells were incubated for 6 h at 37 °C with Me_2SO vehicle, 6–60 μM MG115, or 6–60 μM $\Delta 12\text{-PGJ}_2$. Cellular accumulation of polyubiquitinated proteins was determined by Western blot analysis with anti-ubiquitin antibody. *C*, $\Delta 12\text{-PGJ}_2$ causes the accumulation of high molecular weight species of polyubiquitin. The Western blot in *B* was quantitated by densitometry to determine the molecular weight distribution of polyubiquitinated species that accumulated in RKO cells after incubation with Me_2SO (*light gray stippled trace*), 60 μM $\Delta 12\text{-PGJ}_2$ (*solid black trace*), or 20 μM MG115 (*darker gray trace*). *Arrows* indicate the molecular mass of the most abundant polyubiquitin species.

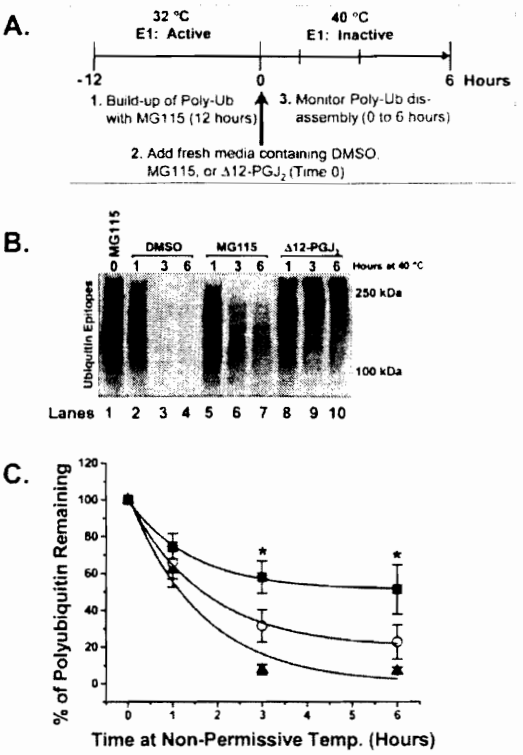

FIG. 7. **$\Delta 12\text{-PGJ}_2$ inhibits polyubiquitin disassembly in ts20 cells.** *A*, schematic representation of the experimental conditions used in the polyubiquitin disassembly assay. *B*, $\Delta 12\text{-PGJ}_2$ inhibits the disassembly of polyubiquitin chains. Ts20 cells were incubated with 1 μM MG115 at 32 °C for 12 h. Under these conditions, the temperature-sensitive E1 ubiquitin-activating enzyme is active, and ts20 cells accumulate polyubiquitinated proteins. After the initial incubation, fresh medium was added containing either Me_2SO, MG115 (6 μM), or $\Delta 12\text{-PGJ}_2$ (20 μM), and the ts20 cells were incubated for an additional 0–6 h at 40 °C. At 40 °C, the E1 enzyme becomes inactivated and ts20 cells stop assembling polyubiquitin, thereby facilitating the measurement of polyubiquitin disassembly. *C*, $\Delta 12\text{-PGJ}_2$ increases the half-life of polyubiquitin in cells. The Western blot in *B* was quantitated by densitometry to determine the half-life ($t_{1/2}$) of polyubiquitin in ts20 cells treated with Me_2SO (▲), 6 μM MG115 (○), or 20 μM $\Delta 12\text{-PGJ}_2$ (■). 100% is defined as the total polyubiquitin at time 0. Data are fit to first-order exponential decay curves and represent the mean ± S.D., $n = 3$ experiments, with statistical significance indicated (*, ANOVA, $p < 0.05$).

activity in this assay. We note that MDL 28170 (180 μM) did not impair the degradation of z-LRGG-MCA under these conditions (data not shown). Furthermore $\Delta 12\text{-PGJ}_2$ (≤180 μM) did not inhibit the degradation of a proteasome substrate, z-LLE-MCA, or a caspase-3 substrate, Ac-DEVD-MCA, indicating selectivity for the inhibition of isopeptidase activity (data not shown).

Due to recent findings, we examined the effects of 4-hydroxynonenal (4-HNE) treatment on the disassembly of polyubiquitin in ts20 cells. Electrophilic prostaglandins can generate oxidized lipids, such as 4-HNE (26–28), which is known to inhibit proteasome activity at millimolar concentrations (31–35). Like $\Delta 12\text{-PGJ}_2$, this compound can form covalent adducts with proteins. If generation of oxidized lipids or covalent modification of target proteins are mechanisms by which $\Delta 12\text{-PGJ}_2$ inhibits the disassembly of polyubiquitinated substrates, then 4-HNE should act in a manner analogous to $\Delta 12\text{-PGJ}_2$. How-

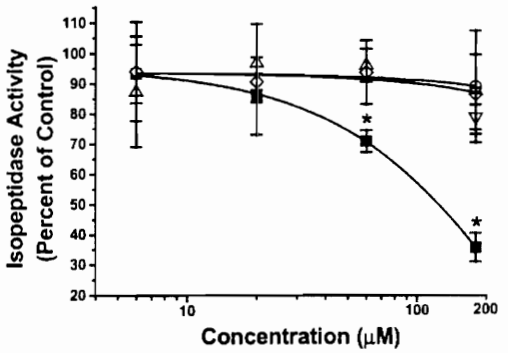

FIG. 8. **Δ12-PGJ$_2$ inhibits isopeptidase activity in RKO cell lysates.** Cell lysates were obtained from RKO cells (0.5 μg/ml) and treated with MG115 (30 μM) to prevent proteasome-mediated degradation of the isopeptidase substrate, z-LRGG-AMC. These lysates were then co-incubated with 6–180 μM Δ12-PGJ$_2$ (■), 6–180 μM PGA$_1$ (△), 6–180 μM PGB$_1$ (◇), 180 μM PGE$_2$ (▽), or 180 μM 15-keto-PGE$_2$ (○) for 0.5 h on ice. Isopeptidase substrate (z-LRGG-AMC) was added, and the reaction mixture was incubated for 3 h at 37 °C. Sample fluorescence was monitored as before. Data are fit to first-order exponential decay curves and represent the mean ± S.D., n = 3 experiments. with statistical significance indicated (*, ANOVA, $p < 0.05$).

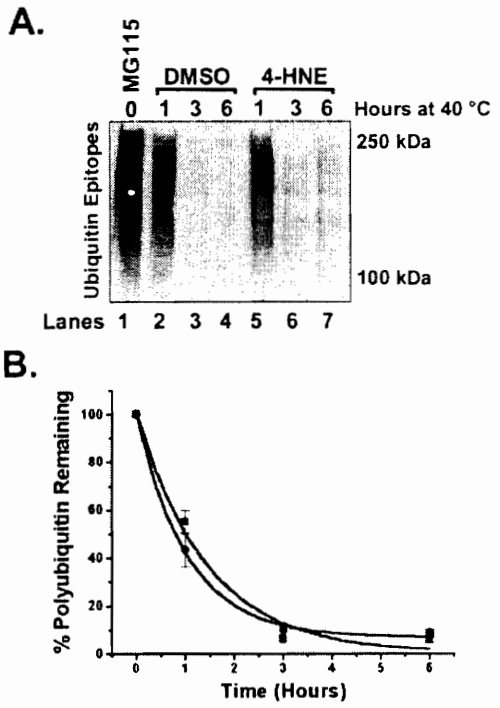

FIG. 9. **4-Hydroxynonenal does not inhibit polyubiquitin disassembly in ts20 cells.** *A*, experimental conditions were set as in Fig. 7. At time 0, medium containing MG115 (1 μM, 12 h) was aspirated from ts20 cells and fresh medium containing either Me_2SO or 4-HNE was added. The cells were then incubated at 40 °C to facilitate the monitoring of polyubiquitin disassembly. *B*, the Western blot in *A* was quantitated by densitometry to determine the extent of polyubiquitin disassembly in ts20 cells treated with Me_2SO (■) or 20 μM 4-HNE (●). 100% is defined as the total polyubiquitin at time 0. Data are the mean ± S.D., n = 3 experiments.

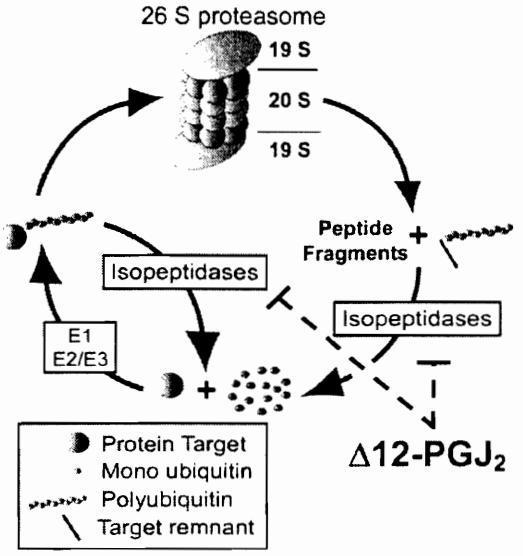

FIG. 10. **Schematic diagram of the ubiquitin-dependent proteasome pathway and site of action of Δ12-PGJ$_2$.**

ever, we did not observe a significant inhibition of polyubiquitin disassembly by 4-HNE (Fig. 9, *lanes 5–7*), even at cytotoxic concentrations, when compared with control (Fig. 9, *lanes 2–4*). Furthermore, 4-HNE (≤180 μM) had no effect on the degradation of the isopeptidase substrate, z-LRGG-AMC (data not shown). Combined, these data suggest that Δ12-PGJ$_2$ does not mediate these effects through oxidized lipids such as 4-HNE.

DISCUSSION

Herein we report that Δ12-PGJ$_2$ causes apoptosis independently of p53-mediated transcription. We also provide evidence that Δ12-PGJ$_2$ inhibits the proteasome pathway via a novel mechanism: inhibition of cellular ubiquitin isopeptidase activity (Fig. 10). Proteasome pathway inhibitors can induce apoptosis independently of p53 (36, 37), suggesting that inhibition of cellular isopeptidase activity is a mechanism by which Δ12-PGJ$_2$ causes apoptosis.

Inhibition of cellular ubiquitin isopeptidase activity should have five predictable effects. First, polyubiquitin and/or polyubiquitinated proteins should accumulate (Figs. 5, *B—D*, and 6*B*). Second, the cellular pool of monomeric ubiquitin should diminish and its depletion should slow the rate of ubiquitin-protein conjugation, leading to accumulation of unmodified protein (*e.g.* p53) (Fig. 5*A*). Third, cells with active 26 S proteasome and inactive isopeptidases (*i.e.* Δ12-PGJ$_2$ treatment) should retain polyubiquitin-protein conjugates differing in composition compared with cells with active isopeptidases and inactive 26 S proteasome (*i.e.* MG115 treatment) (Fig. 6*C*). Fourth, the rate of polyubiquitin disassembly by isopeptidases should slow, increasing the half-life of polyubiquitin conjugates (Fig. 7). Last, the cleavage of peptide substrates by isopeptidases should be inhibited (Fig. 8). Our experiments affirm each of these predictions.

The exact trigger for apoptosis due to proteasome pathway inhibition is unknown. However, disruption of proteasome pathway function could result in the buildup of detrimental proteins (*i.e.* pro-apoptotic or damaged proteins). Also, as others and we have shown, this disruption alters monoubiquitin/

polyubiquitin dynamics, which could affect essential processes that rely on ubiquitin modification for function. Thus, isopeptidase inhibition by Δ12-PGJ$_2$ could cause apoptosis by these and/or other mechanisms.

Δ12-PGJ$_2$ is the first isopeptidase inhibitor reported to exert its effects in intact cells. Nonhydrolyzable ubiquitin dimer analogs (~16 kDa) (38) and ubiquitin aldehyde (~8.5 kDa) (39, 40) inhibitors are suitable for investigation of isolated ubiquitin isopeptidases, but not for investigations with intact cells. We are presently investigating whether Δ12-PGJ$_2$ acts directly, via Michael adduct formation at their active site sulfhydryl, or indirectly by modulating cellular sulfhydryl-disulfide status analogous to the mechanism described by Liu *et al.* (41). We are also investigating whether selective inhibition exists between the various isopeptidase family members.

As a novel modulator of the ubiquitin-dependent proteasome pathway, Δ12-PGJ$_2$ differs chemically and mechanistically from lactacystin (42), eponemycin (43), nitric oxide (44), 4-hydroxynonenal (45), and peptide-aldehyde or boronate inhibitors (26), all of which slow the disassembly of ubiquitin-protein conjugates by irreversibly modifying the 20 S catalytic subunit of the 26 S proteasome. The fact that agents as chemically diverse as these are each apoptotic agonists reinforces the proposition that the ubiquitin-proteasome pathway contains molecular targets for antineoplastic drug development (26). Importantly, our results suggest that inhibition of the isopeptidase component of this pathway causes apoptosis independently of p53-mediated gene transactivation; Δ12-PGJ$_2$ inactivated p53-mediated transcription, caused apoptosis, and inhibited cellular isopeptidase activity under identical conditions. Furthermore, it caused apoptosis in cells expressing dysfunctional allelic variants of p53 (46, 47). Agents that cause apoptosis independently of p53 are of considerable interest, because the majority of drugs used in oncology have lower efficacy in cells with mutant p53 compared with cells with wild type p53 (48). The response to chemotherapy is complex, and investigations focused on a single genetic factor, like p53, may exaggerate its role. However, numerous investigations show that disruption of p53 impairs the efficacy of p53-dependent drugs, *e.g.* 5-fluorouracil, used for a range of oncological disorders (49–53).

Δ12-PGJ$_2$ can originate from rapid dehydration of its parent compound, PGD$_2$ (29, 54). We conclude that the cross-conjugated dienone (Δ9,Δ12-olefin flanking the C11 carbonyl) accounts for the activity we describe. Naturally occurring A series PG with a single endocyclic α,β-unsaturated ketone are not as efficacious as apoptotic agonists and isopeptidase inhibitors, compared with Δ12-PGJ$_2$. It is noteworthy that endogenous formation of PGD$_2$, the parent of Δ12-PGJ$_2$, correlates inversely with the metastatic potential of murine malignant melanoma cells (55, 56). Although we originally attributed this beneficial effect to PGD$_2$ and its inhibition of platelet-tumor cell aggregates, it is plausible that Δ12-PGJ$_2$ might also have contributed as an apoptotic agonist.

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CHAPTER 3

PHARMACOPHORE MODEL FOR NOVEL INHIBITORS OF UBIQUITIN ISOPEPTIDASES THAT INDUCE P53-INDEPENDENT CELL DEATH

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Pharmacophore Model for Novel Inhibitors of Ubiquitin Isopeptidases That Induce p53-Independent Cell Death

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ABSTRACT

The tumor suppressor p53 is mutated in more than 50% of all cancers. Importantly, most clinically useful antineoplastic agents are less potent and efficacious in the context of mutant p53. This situation has prompted a search for agents that cause tumor cell death via molecular mechanisms independent of p53. Our recent investigations with electrophilic prostaglandins enabled us to devise a pharmacophore and mechanism of action hypothesis relevant to this problem: a cross-conjugated α,β-unsaturated dienone with two sterically accessible electrophilic β-carbons is a molecular determinant that confers activity among this class of ubiquitin isopeptidases inhibitors, and that inhibitors of ubiquitin isopeptidases cause cell death in vitro independently of p53. Here, we report the use of the National Cancer Institute's Developmental Therapeutics Database to identify compounds to test this hypothesis. Shikoccin (a diterpene), dibenzylideneacetone, and curcumin fit the pharmacophore hypothesis, inhibit cellular isopeptidases, and cause cell death independently of p53 in isogenic pairs of RKO and HCT 116 cells with differential p53 status. The sesquiterpene achillin and 2,6-diphenyl-4*H*-thiopyran-4-one, which have cross-conjugated dienones with sterically hindered electrophilic β-carbons, do not inhibit isopeptidases or cause significant cell death. Furthermore, we show that a catalytic-site proteasome inhibitor causes cell death independently of p53. Combined, these data verify the p53-independence of cell death caused by inhibitors of the proteasome pathway and support the proposition that the ubiquitin-dependent proteasome pathway may contain molecular targets suitable for antineoplastic drug discovery.

Ubiquitin isopeptidases (ubiquitin specific proteases) are a family of cysteine proteases that salvage ubiquitin for its reuse by the 26S proteasome system (Hochstrasser, 1996; Hershko and Ciechanover, 1998) and regulate the activity of a variety of substrates by altering their ubiquitination status. The ubiquitin salvage activity of the isopeptidases maintains a cellular pool of monomeric ubiquitin by cleaving the isopeptide bond between the C-terminal carboxyl of ubiquitin and the ϵ-amino group of a lysine residue on an adjacent protein, thereby disassembling ubiquitin oligomers, ubiquitin-protein conjugates, and ubiquitin-peptide conjugates. Few inhibitors of isopeptidases have been identified, other than analogs based on ubiquitin itself. These include nonhydrolyzable ubiquitin dimer analogs (~16 kDa) (Yin et al., 2001) and ubiquitin aldehyde (~8.5 kDa) (Dang et al., 1998), which are suitable for investigating isolated enzymes. Recently, we reported that Δ12-prostaglandin J_2 (Δ12-PGJ_2) is a novel isopeptidase inhibitor with activity in intact cells. Results with cyclopentenone prostaglandins (PG) prompted our hypothesis that isopeptidase inhibition depends on nuances of olefin-ketone conjugation. For example, Δ12-PGJ_2, with its cross-conjugated α,β-unsaturated dienone substituent and two sterically accessible β-carbons (Rodriguez et al., 1997), was a potent inhibitor of isopeptidase activity. PGA_1, PGA_2, and 15-keto-PGs with a simple α,β-unsaturated ketone and only one accessible β-carbon were significantly less potent. PGB_1 with an α,β-unsaturated ketone and a sterically hindered β-carbon was inactive (Mullally et al., 2001). In its current formulation, our pharmacophore hypothesis predicts that compounds chemically unrelated to PG, but with a cross-conjugated α,β-unsaturated ketone and two sterically accessible β-carbons, will also inhibit ubiquitin isopeptidases. Although it disrupts ubiquitin salvage and impairs protein turnover, Δ12-PGJ_2 induces apoptosis independently of tumor suppressor p53 *trans*-activation (Mullally

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ABBREVIATIONS: PG, prostaglandin; DBA, dibenzylideneacetone; DMEM, Dulbecco's minimum essential medium; NSC-302979, shikoccin; NSC-156236, achillin; MG115, carbobenzyloxy-L-leucyl-L-leucyl-norvaline; z-LLVY-MCA, succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine α-(4-methyl-coumaryl-7-amide); z-LRGG-MCA, carbobenzoxy-L-leucyl-L-arginyl-L-glycyl-L-glycine α-(4-methyl-coumaryl-7-amide); MTT, (3-(4,5-dimethylthiazo)-2-yl)-2,5-diphenyltetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; UB, ubiquitin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; NCI DTP, National Cancer Institute Developmental Therapeutics Program; DPTP, 2,6-diphenyl-4*H*-thiopyran-4-one; NSC-32982, curcumin; DMSO, dimethyl sulfoxide.

et al., 2001). Therefore, our corresponding pharmacological mechanism hypothesis predicts that such isopeptidase inhibitors will also induce cell death independently of tumor suppressor p53 function. Herein, we report the identification of dibenzylideneacetone (DBA), curcumin, and, via the National Cancer Institute's Developmental Therapeutics Database, a diterpene, shikoccin (NSC-302979), as agents that fulfill these predictions and reinforce our pharmacophore and mechanism hypotheses. As isopeptidase inhibitors, DBA, curcumin, and NSC-302979 inhibit the proteasome pathway in a manner chemically and mechanistically distinct from lactacystin (Fenteany et al., 1995), eponemycin (Meng et al., 1999), and peptide-aldehyde or boronate inhibitors (Adams et al., 1999), which all covalently inhibit the 20S catalytic subunit of the proteasome. Our results support the hypothesis that the sterically accessible, cross-conjugated α,β-unsaturated dienone is a pharmacophore that confers inhibitory activity toward isopeptidases. Furthermore, these results lend support to the proposition that the ubiquitin-dependent proteasome pathway contains molecular targets suitable for antineoplastic drug discovery (Kisselev and Goldberg, 2000).

Experimental Procedures

Materials. We used Δ12-PGJ$_2$ and PGB$_1$ (Cayman Chemicals, Ann Arbor, MI); dibenzylideneacetone, etoposide, paclitaxel, Curcumin, and 2,6-diphenyl-4*H*-thiopyran-4-one (Sigma, St. Louis, MO); NSC-302979 and NSC-156236 (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute); complete protease inhibitor mixture (Roche Applied Science, Indianapolis, IN); enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ); antibodies directed against p53 (DO-1), horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and ubiquitin (Zymed Laboratories, Inc., San Francisco, CA); MG115 (Peptides International, Louisville, KY); z-LLVY-MCA, z-LRGG-MCA (Biomol Research Laboratories, Plymouth Meeting, PA); (3-(4,5-dimethylthiazo)-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Molecular Probes Inc., Eugene, OR); ubiquitin-PEST (Ub-PEST; a gift of Dr. Martin Rechsteiner, Department of Biochemistry, University of Utah, Salt Lake City, UT). Centricon YM-30 centrifugal filters (Amicon Bioseparations; Millipore, Bedford, MA).

Cell Culture. We used RKO and RKO-E6 colon cancer cells (gift from Dr. Mark Meuth, Institute for Cancer Studies, University of Sheffield, Sheffield, UK). We used HCT 116 colon cancer cells with varying degrees of p53 haplosufficiency (gift of Dr. Bert Vogelstein, Johns Hopkins School of Medicine, Baltimore, MD). We maintained RKO and RKO-E6 cells in DMEM [supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin and streptomycin, and 10% (v/v) fetal bovine serum] in a humidified incubator with 5% CO_2. We maintained HCT 116 cells in McCoy's 5A medium [supplemented with 1 mM sodium pyruvate, 50 units/ml penicillin and streptomycin, and 10% (v/v) fetal bovine serum] in a humidified incubator with 5% CO_2.

Immunochemical Detection of Proteins. We removed the medium and lysed cells in 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM EDTA with 0.1% SDS, 0.1% deoxycholate, 1× complete protease inhibitor mixture. We measured protein concentration by the method of Bradford (1976). We fractionated equal portions of the total cell lysate from each sample (12.5 μg of protein) by SDS-PAGE. We transferred proteins to polyvinylidene difluoride blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline [20 mM Tris-HCl, pH 7.5, 100 mM sodium chloride, 0.1% (v/v) Tween 20]. We detected proteins immunochemically by using primary antibodies directed against p53 (1:4000) or ubiquitin (1:1000), followed by horseradish peroxidase-conjugated secondary antibodies (1:4000). We detected antigen-antibody complexes with enhanced chemiluminescence reagents. We scanned gels and quantified intensities using Kodak 1D Image Analysis Software (Eastman Kodak, Rochester, NY).

Cell Viability Assay. We determined cell viability by the MTT assay. Briefly, we incubated 1×10^5 cells per well of a sterile, 96-well assay plate with 0–60 μM test compounds for 48 h. We added MTT reagent to each well (final concentration, 0.5 mg/ml) and incubated for an additional 3 h. We aspirated the media and remaining MTT reagent from each well and added 100 μl of HCl/isopropanol (1:24). We measured the absorbance of each sample at 405 nm.

Ubiquitin Isopeptidase Activity Assays. We measured cellular isopeptidase enzymatic activity with two assays that used different substrates. In one assay we used Ub-PEST, a full-length ubiquitin molecule with an 18-amino acid C-terminal peptide extension (total mass, 10.5 kDa). Ubiquitin isopeptidases specifically cleave the 18-amino acid peptide extension, releasing full-length ubiquitin (8.5 kDa). Briefly, we incubated 6×10^5 cells with 0–60 μM test compounds for 12 h. We lysed cells in 50 μl of 25 mM HEPES, 5 mM EDTA, 0.1% CHAPS, 5 mM ATP, pH 7.5. We adjusted the protein concentration of each sample to 0.3 mg/ml and incubated with 50 μg/ml Ub-PEST for 45 min at 25°C. Under these conditions Ub-PEST hydrolysis occurs at a linear rate. We mixed 20-μl samples with 20 μl of 2× Laemmli buffer, boiled briefly, and fractionated by SDS-PAGE. We monitored isopeptidase activity by determining the amount of product (8.5-kDa ubiquitin) formation.

The second assay used a fluorescent tetrapeptide, z-LRGG-AMC, as a substrate that mimics the carboxyl terminus of ubiquitin. Isopeptidase activity hydrolyzes the bond between the c-terminal glycine and the fluorophore. This tetrapeptide also undergoes slow proteolysis by the catalytic subunit of the proteasome. To minimize this background rate of proteolysis, we incubated cell lysates with 30 μM MG115 for 30 min at 4°C, before substrate incubation (>90% proteasome inhibition). We treated cells as above, lysed them in 250 μl of lysis buffer per sample, and adjusted their protein concentration to 0.5 mg/ml before incubation with MG115. We then added z-LRGG-AMC substrate and quantified fluorescence of the AMC moiety cleaved by isopeptidase action.

We determined whether Δ12-PGJ$_2$ acted irreversibly. We incubated cell lysates with vehicle or 100 μM test compound and measured isopeptidase activity fluorometrically. We dialyzed a 500-μl portion of each sample through a Centricon filter with a molecular mass cut-off of 25 kDa. After washing each sample with 3 volumes of assay buffer, we measured the isopeptidase activity in the filtrate. If filtration did not reverse inhibition, it implies that Δ12-PGJ$_2$ is an irreversible inhibitor.

Statistics. We used analysis of variance for statistical calculations.

Results

SubStructure Analysis of the NCI Cancer Screening Database. The Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI) has systematically evaluated >70,000 compounds for cytostatic and cytotoxic activity against human cell lines in vitro (Monks et al., 1997). The cell lines typify cancers of the colon, blood (leukemia), brain, breast, kidney, lung, ovary, prostate, and skin (melanoma). Intramural NCI investigators, who have access to the entire database, have applied this information-intensive approach with promising results (Weinstein et al., 1997; Shi et al., 1998). Extramural investigators have access to a restricted portion of the database, last released in August 2000. Using these available data, we conducted substructure searches to test our hypothesis that the cross-conjugated α,β-unsaturated dienone with two sterically accessible β-carbons is the primary molecular determinant that confers the

inhibition of isopeptidases. Specifically, we sought nonprostanoid compounds with this feature that varied in the accessibility of their olefinic β-carbons (e.g., β-carbons with -H versus with $-CH_3$ substituents).

Our substructure query, 2-cyclopenten-5-methylene-1-one (Fig. 1, i), yielded eight compounds. All have a cyclic (bis) α,β-unsaturated ketone with one *endo*- and one *exo*-olefin. They are otherwise chemically unrelated to Δ12-PGJ_2 or other PGs. The NCI provided two of the eight compounds we requested for our experimental use: the sesquiterpene NSC-156236 (Fig. 1, ii) and the diterpene NSC-302979 (Fig. 1, v). Like Δ12-PGJ_2 (Fig. 1, vi), the *endo*- and *exo*-olefins of NSC-302979 have sterically accessible β-carbons that can react with nucleophiles (e.g., cysteine; Rodriguez et al., 1997). Analogous to PGB_1 (Fig. 1, iii), NSC-156236 has methyl-substituted β-carbons at the *endo*- and the *exo*-olefin of the dienone. These β-carbons are sterically hindered and therefore should not react readily with relevant physiological nucleophiles (Rodriguez et al., 1997). To further reinforce our pharmacophore hypothesis, we also evaluated several commercially available compounds. Two of these compounds, DBA (Fig. 1, vii) and curcumin (Fig. 1, viii), have sterically accessible β-carbons. The final compound, 2,6-diphenyl-4*H*-thiopyran-4-one (DPTP; Fig. 1, iv), resembles DBA, except that it has a bulky sulfur atom sterically hindering its β-carbons. In summary, our pharmacophore hypothesis predicts that compounds ii through iv will be inactive as isopeptidase inhibitors and compounds v through viii will be active as isopeptidase inhibitors.

Electrophilic Cross-Conjugated Dienones Inhibit Cellular Ubiquitin Isopeptidases. Little is known about the substrate specificity of the individual isopeptidase family members. To investigate total cellular isopeptidase activity, we use two simple substrates, Ub-PEST and z-LRGG-AMC, which most isopeptidases use as substrates. Figure 2A shows the effect of the test panel compounds, Δ12-PGJ_2 (lanes 3–5), DBA (lanes 6–8), NSC-302979 (lanes 9–11), PGB_1 (lane 12), NSC-156236 (lane 13), curcumin (lanes 15–17), and DPTP (lane 18) on cleavage of the Ub-PEST substrate by isopeptidases in HCT 116 colon cancer cell lines. Similar results were obtained for RKO cells (raw data not shown). Consistent with our pharmacophore hypothesis, the compounds with cross-conjugated ketones and sterically accessible β-carbons, Δ12-PGJ_2, DBA, NSC-302979, and curcumin, each inhibited isopeptidase activity in a concentration-dependent manner (Fig. 2B). Compounds with sterically hindered β-carbons (PGB_1, NSC-156236, and DPTP) did not inhibit isopeptidase activity. The rank order of potency for inhibition of ubiquitin-PEST hydrolysis by isopeptidases was DBA ≈ NSC-302979 ≥ Δ12-PGJ_2 > curcumin ≫ NSC-156236 ≈ PGB_1 ≈ DPTP.

To verify the results with Ub-PEST in Fig. 2, z-LRGG-AMC was used as the substrate for isopeptidases (Fig. 3). NSC-302979, Δ12-PGJ_2, DBA, and curcumin each inhibited

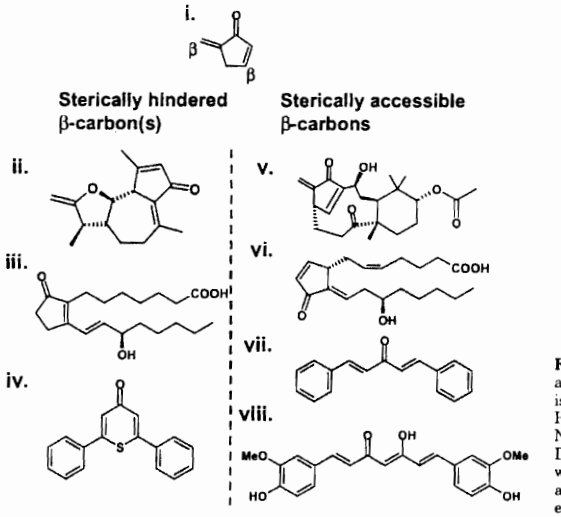

Fig. 1. Chemical structures of the pharmacophore test panel. We used 2-cyclopenten-5-methylene-1-one (i) as a substructure to search the NCI-DTP database. We identified, and the NCI provided, NSC-156236 (achillin, ii) and NSC-302979 (shikoccin, v). We also identified and used PGB_1 (iii), DPTP (iv), Δ12-PGJ_2 (vi), DBA (vii), and curcumin (viii) to test our pharmacophore hypothesis.

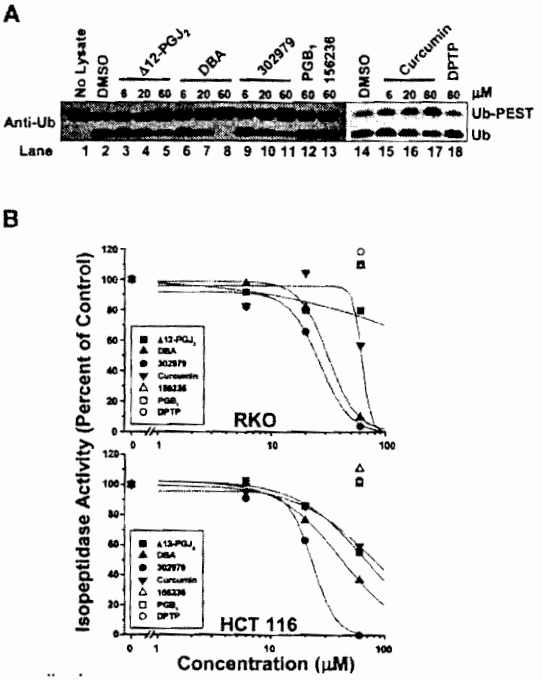

Fig. 2. Effect of the pharmacophore test panel on ubiquitin isopeptidase activity in colon cancer cells: Ub-PEST as substrate. A, inhibition of isopeptidase proteolysis of Ub-PEST substrate. We treated RKO and HCT 116 cells with DMSO vehicle, 6, 20, or 60 μM Δ12-PGJ_2, DBA, NSC-302979, or curcumin, 60 μM PGB_1, 60 μM NSC-156236, or 60 μM DPTP for 12 h at 37°C. We incubated cell lysates from each treatment with Ub-PEST as described under *Materials and Methods*. We fractionated samples by SDS-PAGE and detected proteins with ubiquitin epitopes immunochemically (HCT 116 $^{+/+}$ Western blot shown as example of raw data). Lane 1 shows the substrate before incubation with lysate. B, inhibition of isopeptidase activity by test panel compounds. We determined isopeptidase activity by measuring, via densitometry, the amount of Ub generated by isopeptidase cleavage of Ub-PEST. The amount of Ub generated in the vehicle treated sample (A, lane 2) is arbitrarily designated 100%.

ubiquitin isopeptidase activity, whereas NSC-156236, PGB_1, and DPTP did not. The rank-order of potency for inhibition of z-LRGG-AMC hydrolysis by ubiquitin isopeptidases was NSC-302979 > DBA > Δ12-PGJ_2 > curcumin ≫ NSC-156236 ≈ PGB_1 ≈ DPTP.

Consistent with inhibition of the isopeptidases that disassemble ubiquitin polymers and ubiquitin-protein conjugates, protein species with polyubiquitin conjugation accumulated in cells (e.g., in RKO-E6 cells, Fig. 4A) treated with Δ12-PGJ_2 (lanes 2–4), NSC-302979 (lanes 5–7), DBA (lanes 8–10) or curcumin (lanes 14–16). PGB_1 (lane 11), NSC-156236 (lane 12), and DPTP (lane 17), compounds that did not inhibit isopeptidase activity, did not cause appreciable cellular accumulation of ubiquitin conjugates.

As a consequence of polyubiquitin accumulation, monoubiquitin is initially depleted in cells treated with isopeptidase inhibitors [8.5-kDa band, Fig. 4A, lanes 2, 5, 8, and 14 versus lanes 1 or 13). We predicted that this would affect the rate at which substrates are degraded via the ubiquitin-proteasome pathway. RKO and RKO-E6 cells allow a convenient test of our pharmacophore hypothesis with p53, a single, explicit substrate targeted for ubiquitin-dependent proteolysis.

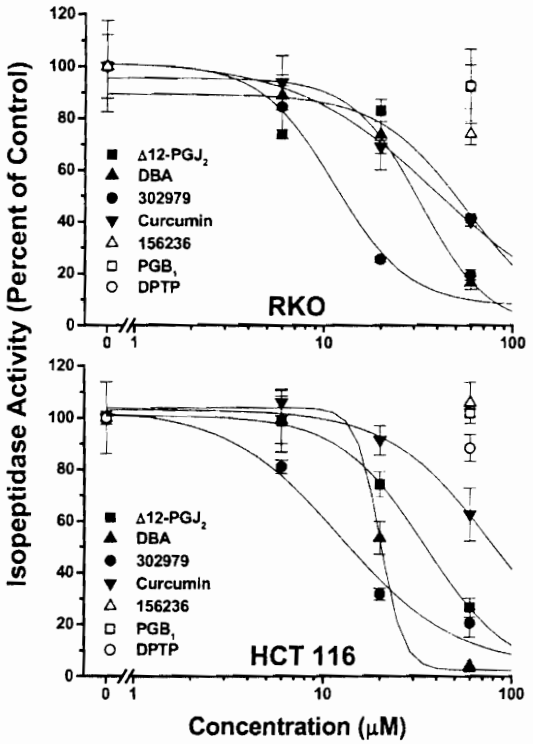

Fig. 3. Effect of the pharmacophore test panel on ubiquitin isopeptidase activity in colon cancer cells: z-LRGG-AMC as substrate. We treated RKO (upper) and HCT $116^{+/+}$ cells (lower) with DMSO vehicle, 6, 20, or 60 μM Δ12-PGJ_2, DBA, NSC-302979, or curcumin, 60 μM PGB_1, 60 μM NSC-156236, or 60 μM DPTP for 12 h at 37°C. We incubated cell lysates (0.5 mg/ml) from each treatment with the isopeptidase substrate, z-LRGG-AMC, for 3 h at 37°C. We determined the amount of AMC cleaved by isopeptidase fluorometrically, as described under *Materials and Methods*.

RKO-E6 cells, an isogenic variant of RKO cells, harbor the HPV-E6 oncoprotein that, together with E6-AP ubiquitin ligase, hastens proteasome-mediated degradation of p53 (Fig. 4B, i; Ashcroft and Vousden, 1999). Thus, under most conditions, the levels of p53 are significantly lower in RKO-E6 cells than in RKO cells (e.g., treatment of cells with vehicle or etoposide, a DNA damaging agent; Fig. 4B, ii, lanes 1 and 2, respectively, and iii). One exception is when the ubiquitin-proteasome pathway is inhibited (e.g., treatment of cells with the proteasome inhibitor, MG115; lane 3); then, accumulation of p53 in RKO-E6 and RKO cells equalizes. Accordingly, the ratio of p53 protein (RKO-E6 cells/RKO cells) approached unity in cells treated with the isopeptidase inhibitors, Δ12-PGJ_2 (lane 4), NSC-302979 (lane 5), DBA (lane 6), and curcumin (lane 9). Test compounds that did not inhibit ubiquitin isopeptidase activity [PGB_1 (lane 7), NSC 156236 (lane 8), and DPTP (lane 10)] had p53 protein ratios (RKO-E6:RKO) significantly less than unity, suggesting that they do not inhibit p53 degradation via the ubiquitin-proteasome pathway. p53 accumulation caused by the pharmacophore test compounds is not a result of 20S proteasome inhibition, because none of the compounds with cross-conjugated dienones inhibited the 20S catalytic subunit of the proteasome under these conditions (Mullally et al., 2001; and data not shown). Note that despite its accumulation in the presence of Δ12-PGJ_2, p53 is inactivated as a transcription factor under these conditions (Mullally et al., 2001); furthermore, p53 activation is insufficient to cause its stabilization in RKO-E6 cells, because the p53 protein ratio (RKO-E6:RKO = 0.1) in etoposide treated cells fails to approach unity. Collectively, the data in Figs. 2 through 4 affirm that our pharmacophore hypothesis extends beyond the prostanoid family into the diterpene and other chemical families.

The Prototype Isopeptidase Inhibitor, Δ12-PGJ_2, Inhibits Ubiquitin Isopeptidases Irreversibly. Although we have not yet identified a covalent complex between an isopeptidase and one of the isopeptidase inhibitors, this proposed mechanism of action is consistent with data that we have obtained from analyzing cell lysates treated with Δ12-PGJ_2. Treatment of cell lysates (versus treatment of whole cells) should exclude the likelihood of transcriptional/translational events due to the nature of the lysate preparation (i.e., sonication of cell lysates probably shears all polynucleotides). Δ12-PGJ_2 inhibited isopeptidase activity in treated cell lysates (Fig. 5, predialysis). Furthermore, isopeptidase inhibition by Δ12-PGJ_2 could not be reversed by dialysis of treated lysates (Fig. 5, postdialysis).

Inhibitors of Ubiquitin Isopeptidases Cause Cell Death Independently of Tumor Suppressor p53 Function. Previously, we showed that electrophilic prostaglandins, typified by Δ12-PGJ_2, inhibit p53-mediated transcription under the same conditions in which they cause cell death, suggesting that cell death occurs independently of p53 (Mullally et al., 2001). Therefore, integration of our pharmacophore and molecular mechanism hypotheses predicts that various isopeptidase inhibitors will cause cell death independently of tumor suppressor p53 function. Analysis of the NCI 60 cell line cancer screening data, according to O'Connor et al. (1997), showed that NCI-302979 and curcumin (NSC-32982) act independently of p53 (Table 1). NSC-156236 had no appreciable cytotoxic activity. Thus, data on NSC-302979, NSC-32892, and NSC-156236, available from the DTP public

database, fulfill the minimal, initial prediction of our hypotheses.

We confirmed that isopeptidase inhibitors caused cell death independently of p53 by investigating their effects on two pairs of isogenic colon cancer cell lines. Isogenic HCT $116^{+/+}$ and HCT 116−/− cell lines have varying degrees of p53 haplosufficiency, $p53^{+/+}$ and p53−/−, respectively (Bunz et al., 1999). NSC-302979, DBA, Δ12-PGJ_2, and curcumin each caused cell death with equal potency (concentration for half-maximal effect) and efficacy (maximal effect) in HCT $116^{+/+}$ cells that are homozygous for p53 and HCT 116−/− cells that are null for p53 (Fig. 6, right hand). NSC-156236, PGB_1, and DPTP, which did not inhibit ubiquitin isopeptidase activity at concentrations <60 μM did not cause significant cell death in HCT $116^{+/+}$ or HCT $116^{-/-}$ cells. Isogenic RKO and RKO-E6 cells accumulate p53 to varying degrees after genomic stress due to the enhancement of p53 ubiquitination by HPV-E6. NSC-302979, DBA, Δ12-PGJ_2, and curcumin each caused cell death with equal potency and efficacy in RKO and RKO-E6 cells (Fig. 6, left). NSC-156236 PGB_1, and DPTP did not cause significant cell death in RKO or RKO-E6 cells.

As procedural controls and for calibration, we evaluated etoposide and paclitaxel on these same pairs of cells. Etoposide typifies agents that cause cell death via a p53-dependent pathway (Lowe et al., 1993). Accordingly, its potency in HCT 116−/− cells was ~4-fold less than its potency in HCT $116^{+/+}$ cells (Fig. 7, top right). Similarly, etoposide potency in RKO-E6 cells was ~3-fold less than its potency in RKO cells (Fig. 7, top left). Paclitaxel typifies an agent that causes cell death via a p53-independent pathway (O'Connor et al., 1997). Although its efficacy (maximal effect observed) in the HCT $116^{-/-}$ and RKO-E6 cells with dysfunctional p53 exceeded its efficacy in the corresponding HCT $116^{+/+}$ and RKO cells, the potency of paclitaxel (concentration for half-maximal effect) was equivalent in HCT $116^{-/-}$ compared with HCT $116^{+/+}$ cells, as well as in RKO-E6 compared with RKO cells (Fig. 7, middle). Lastly, we evaluated MG115, an inhibitor of the 20S catalytic subunit of the proteasome, to compare its effects with isopeptidase inhibitors. There are conflicting reports on the role of p53 in cell death caused by proteasome inhibitors like MG115 (Dietrich et al., 1996; Shinohara et al., 1996; Lopes et al., 1997; Adams et al., 1999; Wagenknecht et al., 1999; An et al., 2000). MG115 caused cell death with equal potency and efficacy in HCT $116^{+/+}$ cells versus HCT $116^{-/-}$ and in RKO-E6 cells versus RKO cells, analogous to isopep-

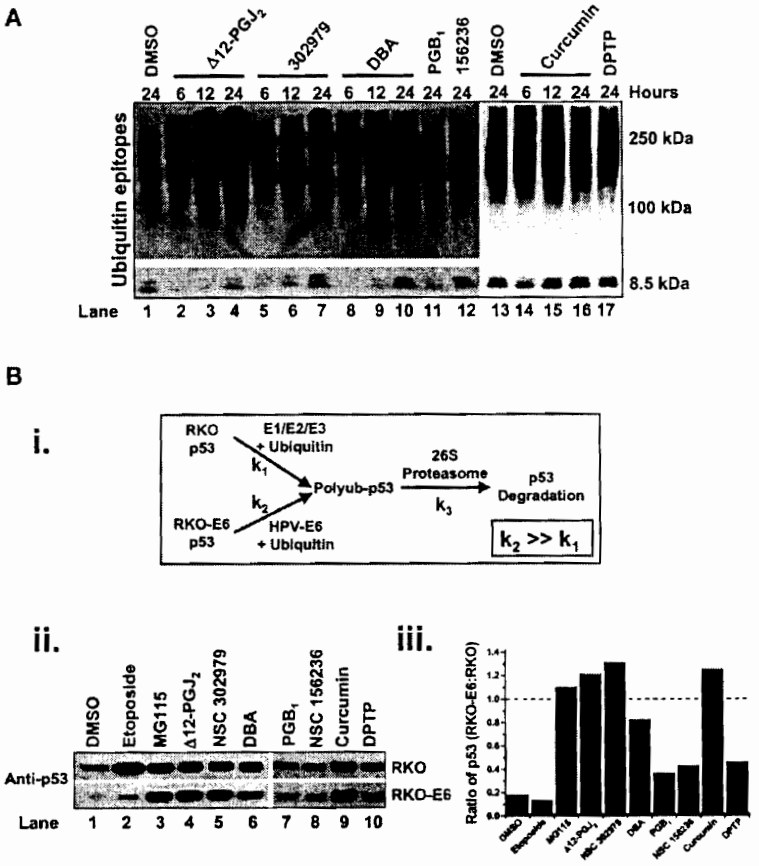

Fig. 4. Effect of the pharmacophore test panel on ubiquitin-dependent proteolysis in colon cancer cells. A, accumulation of polyubiquitin. We treated RKO-E6 cells with DMSO vehicle for 24 h, 37°C, or with the concentrations of test compounds that gave 80% cell death (as determined by cell viability assay at 48 h) for 6, 12, or 24 h at 37°C [EC_{80}, Δ12-PGJ_2 (13.2 μM), DBA (12.0 μM), NSC-302979 (3.5 μM), or curcumin (17 μM)]. For compounds that were inactive in the cell viability assay, we treated with the highest concentrations examined (60 μM PGB_1, NSC-156236, and DPTP for 24 h at 37°C). We fractionated cell lysates by SDS-PAGE and detected proteins with ubiquitin epitopes immunochemically. B, accumulation of p53, a protein targeted for proteasomal degradation. i, Schematic depicting p53 degradation via the proteasome pathway in RKO cells versus RKO-E6 cells. The activity of the HPV E6 oncoprotein hastens p53 degradation in RKO-E6 cells. ii, We treated RKO and RKO-E6 cells with vehicle, etoposide (50 μM), MG115 (20 μM), Δ12-PGJ_2 (60 μM), NSC-302979 (20 μM), DBA (20 μM), PGB_1 (60 μM), NSC-156236 (60 μM), curcumin (60 μM), or DPTP (60 μM) for 6 h, 37°C. We fractionated lysates by SDS-PAGE and determined their p53 content immunochemically. iii, We measured p53 levels by densitometry and calculated the ratio of p53 protein in RKO-E6 cells/RKO cells.

tidase inhibitors (Fig. 7, bottom). Table 2 summarizes the potency of all compounds as cell death agonists in both pairs of cell lines.

Discussion

Our results indicate that nonprostanoid classes of compounds, with α,β-unsaturated ketones and two sterically accessible β-carbons, will inhibit ubiquitin isopeptidase activity. We further demonstrate that these compounds cause cell death independently of p53 tumor suppressor function in vitro. Specifically, the diterpene NSC-302979, the synthetic compound DBA, the prostaglandin Δ12-PGJ$_2$, and the curcuminoid curcumin all cause cell death with efficacy and potency that is indistinguishable ($p > 0.05$) between HCT 116 p53$^{+/+}$ and HCT 116 p53$^{-/-}$ or RKO and RKO-E6 cells. Furthermore, cell death correlated with inhibition of isopeptidase activity. Regression analysis (IC_{50} for inhibition of z-LRGG-AMC hydrolysis by isopeptidase versus IC_{50} for cytotoxicity) yields a straight line with a correlation coefficient $r^2 = 0.93$ ($n = 7$). Regression analysis (IC_{50} for inhibition of ubiquitin-PEST hydrolysis by isopeptidase versus IC_{50} for cytotoxicity) also yields a straight line with $r^2 = 0.73$ ($n = 13$).

Inhibition of ubiquitin isopeptidase activity probably propagates cell death by shifting the polyubiquitin chain length equilibrium to one of greater molecular mass. As a consequence of unfettered polyubiquitin chain growth, the pool of monoubiquitin diminishes. Alteration of monoubiquitin/polyubiquitin dynamics inevitably affects several transcription factors other than p53 (Desterro et al., 2000). Furthermore, with depleted monoubiquitin pools, cells are hampered in their efforts to rid themselves of damaged/toxic proteins, eventually affecting protein-protein or protein-DNA interactions that modulate cell survival and apoptosis. Although our data support a covalent mechanism, we are presently investigating whether α,β-unsaturated dienones covalently inhibit isopeptidases, specifically, via their electrophilic β-carbons

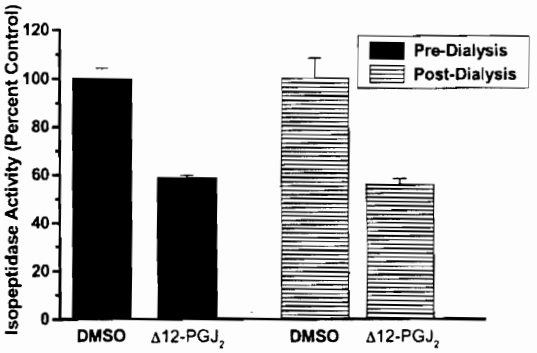

Fig. 5. Reversibility of isopeptidase inhibition by Δ12-PGJ$_2$. We treated cell lysates (0.5 mg/ml) with DMSO vehicle or 100 μM Δ12-PGJ$_2$ for 1 h at 25°C. We divided each of the treated lysates into two aliquots. We immediately analyzed one aliquot for isopeptidase activity by incubating with z-LRGG-AMC as described previously (PreDialysis). We dialyzed the second aliquot by washing with 3 volumes of assay buffer on a centricon YM-30 column, followed by analysis for isopeptidase activity by incubating with z-LRGG-AMC (PostDialysis).

TABLE 1
Mean LC_{50} of NCI DTP database compounds examined

Compound	Mean LC_{50}		Statistical Significance
	Wild Type p53	Mutant p53	
	μM		
NSC-302979	5.4 ± 10^{5a}	6.6 ± 10^{6b}	Indistinguishable ($p \gg 0.05$)
Curcumin	6.1 ± 10^{5a}	6.8 ± 10^{6b}	Indistinguishable ($p \gg 0.05$)
NSC-156236	Inactive	Inactive	

[a] Results from 18 cell lines with wild-type p53.
[b] Results from 41 to 42 cell lines with mutant/deleted p53.

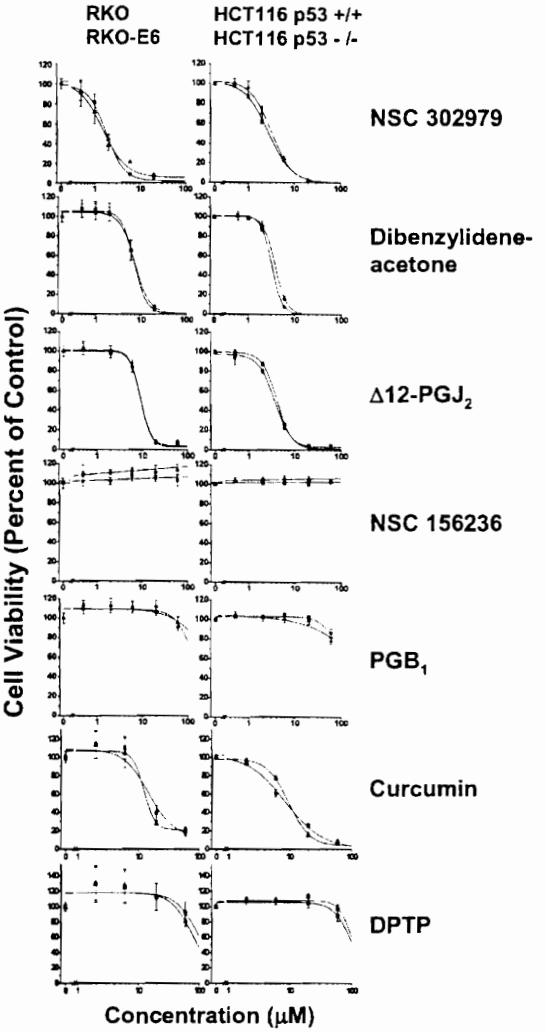

Fig. 6. Cytotoxicity of the pharmacophore test panel in HCT 116 and RKO cell lines with different p53 status. We incubated RKO and RKO-E6 cells (left column), and HCT116 p53$^{+/+}$ and HCT116 p53 $^{-/-}$ cells (right column) with vehicle or 0.5–60 μM NSC-302979, DBA, Δ12-PGJ$_2$, NSC-156236, PGB$_1$, curcumin, or DPTP for 48 h at 37°C. We measured cell viability with MTT reagent as described under *Materials and Methods*. Data are percentage of control viability, mean ± S.D. ($n = 4$).

(e.g., Michael adduct formation between an isopeptidase cysteine residue and the β-carbon of a dienone). Note that compounds with sterically inaccessible or inert β-carbons (NSC-156236, PGB_1, and DPTP) were inactive as isopeptidase inhibitors.

We used the substructure search capabilities of the NCI DTP database (60 cell-line screen) to identify NSC-302979 and NSC-156236, compounds used to test our pharmacophore and mechanism of action hypotheses. We think our results, along with the results by NCI scientists (O'Connor et al., 1997; Weinstein et al., 1997; Shi et al., 1998), exemplify the potential of this database and compound repository and the foresight of the NCI Developmental Therapeutics Branch. Others have suggested that the database content is misaligned with the goal to discover new anticancer drugs, based on a poor correlation between clonogenic survival and the NCI archival antiproliferative activity (Brown, 1997). However, direct extension of data acquired in vitro to clinical situations in vivo is rarely straightforward. Used prudently, to enable or to advance mechanistic and pharmacophore hypotheses, the database supports the quest for anticancer drugs with novel structures and mechanisms of action.

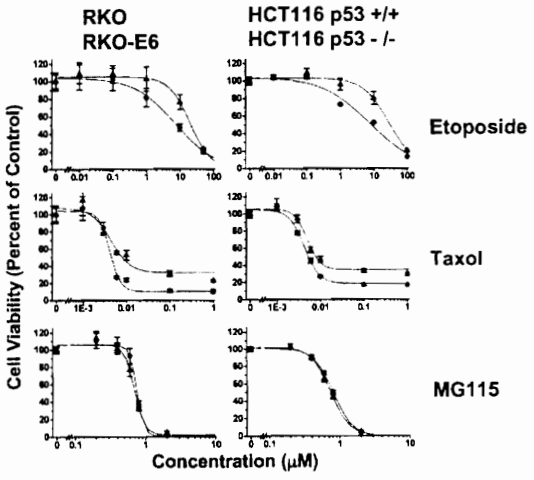

Fig. 7. Cytotoxicity of the calibration set in HCT 116 and RKO cell lines with different p53 status. We incubated RKO and RKO-E6 cells (left column), and HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells (right column) with vehicle, 0.01 to 100 μM etoposide, 0.001 to 1 μM paclitaxel, or 0.02 to 2 μM MG115 for 48 h at 37°C and determined their effect on cell viability, as described under *Materials and Methods*.

Our mechanistic and pharmacophore hypotheses are compatible with the structure-activity relationships reported by Kato et al. (1986), Sasaki et al. (1991) and Sasaki and Fukushima (1994). Kato et al. (1986) reported that Δ12-PGJ_2 and several related Δ7-PGA_1 derivatives (all of which are cross-conjugated dienones) increased the life span of Ehrlich ascites tumor-bearing mice: i.p. doses of 20 to 30 mg/kg/day for 5 consecutive days prolonged survival 66 to 111%. In addition, both Δ12-PGJ_2 and Δ7-PGA_1 exhibit little cross-resistance with cisplatin and doxorubicin in vivo (Sasaki et al., 1991; Sasaki and Fukushima, 1994). Despite these promising results, Δ7-PGA_1 is rapidly metabolized to an inactive compound ($t_{1/2} < 5$ min) in serum (Suzuki et al., 1998). Therefore, our discovery of isopeptidase inhibitors among chemical classes other than PG might be advantageous in surmounting any difficulties intrinsic to the antineoplastic development of the PG class.

There exists considerable debate as to whether agents that inhibit the proteasome pathway cause cell death via a p53-independent process (Dietrich et al., 1996; Shinohara et al., 1996; Lopes et al., 1997; Adams et al., 1999; Wagenknecht et al., 1999; An et al., 2000). Our results with inhibitors of ubiquitin isopeptidase activity and with a representative catalytic subunit inhibitor of the 20S proteasome accord with those who conclude that proteasome inhibition causes apoptosis independently of p53. This debate may originate from faulty assumptions about the competence of p53 that accumulates in cells treated with proteasome pathway inhibitors. For instance, genetically wild-type p53 accumulates in the presence of the isopeptidase inhibitor Δ12-PGJ_2, but in a conformationally and functionally impaired state (Moos et al., 2000; Mullally et al., 2001). An et al. (2000) have also reported that accumulation of wild-type p53 protein and induction of apoptosis occur as independent markers of proteasome inhibition. Therefore, one must use caution when interpreting the consequences of p53 accumulation without first testing its functionality.

The response to chemotherapy is complex; focus on a single factor, no matter how prominent, may exaggerate its role. However, numerous investigations show that disruption of p53 impairs the potency and efficacy of drugs used in oncology [e.g., 5-fluorouracil (Lowe et al., 1994, 1995; Mueller and Eppenberger, 1996; O'Connor et al., 1997; Bunz et al., 1999; Pich, 1998; Weller, 1998; Karpf et al., 2001)]. It is notable that vinca alkaloids, one of the few drug classes that act

TABLE 2
Cytotoxic potency of pharmacophore panel and calibration compounds in HCT 116 and RKO cells with different p53 status

Compound	EC_{50}			
	RKO	RKO-E6	$HCT^{+/+}$	$HCT^{-/-}$
			μM	
NSC-302979	1.8 ± 0.1	1.4 ± 0.1	2.7 ± 0.2	3.2 ± 0.2
DBA	7.0 ± 0.6	6.5 ± 1.1	3.2 ± 0.1	3.8 ± 0.1
Δ 12-PGJ_2	9.0 ± 0.8	9.2 ± 1.0	3.6 ± 0.3	4.1 ± 0.1
Curcumin	12.5 ± 5.9	14.9 ± 8.5	9.7 ± 1.1	8.6 ± 1.3
NSC-156236	>60	>60	>60	>60
PGB_1	>60	>60	>60	>60
DPTP	>60	>60	>60	>60
Etoposide	7.3 ± 1.6	20.0 ± 2.7	7.8 ± 3.9	29.0 ± 6.8
Paclitaxel	0.004 ± 0.0	0.004 ± 0.0	0.004 ± 0.0	0.005 ± 0.0
MG115	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.7 ± 0.0

independently of p53 (O'Connor et al., 1997; Fan et al., 1998), may target the proteasome in addition to tubulin (Piccinini et al., 2001). LDP-341, the first proteasome inhibitor to enter clinical trials, seems to have a favorable safety and efficacy profile (Dalton et al., 2001). Clinical studies to evaluate proteasome inhibition as an adjuvant to systemic chemotherapy are also currently in development (Cusack et al., 2001). Our results demonstrate that another component of the proteasome pathway, isopeptidase activity, warrants further investigation as a target for antineoplastic drug discovery.

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CHAPTER 4

UNPUBLISHED RESULTS AND FUTURE DIRECTIONS

4.1. Unpublished Results

I have provided evidence supporting our hypothesis that certain electrophilic prostaglandins inhibit the ubiquitin-specific protease (USP) activity of the proteasome pathway. Furthermore, I have shown that the cross-conjugated α,β-unsaturated dienone is a molecular determinant for the potency of this activity, and that this chemical feature causes an alteration in cellular ubiquitin dynamics, resulting in accumulation of the polyubiquitin pool, decrease in the monoubiquitin pool, and decrease in the degradation rate of a specific proteasome substrate, p53. In this chapter, using the model isopeptidase inhibitor, Δ12-PGJ$_2$, additional mechanistic evidence will be provided that supports our hypothesis that isopeptidase inhibition by cross-conjugated α,β-unsaturated dienones proceeds via a covalent interaction, and that it is the subsequent inhibition of global cellular protein degradation that ultimately causes cell death. Lastly, in section 4.2, I will discuss some of the future directions that our findings suggest may yield valuable data with regard to proteasome pathway-induced cell death and chronic inflammatory diseases.

4.1.1. Δ12-PGJ$_2$ Does Not Cause Polyubiquitin Accumulation via PPARγ Activation

PGs of the J-series, in particular 15-deoxy-Δ12,Δ14-PGJ$_2$ (15d-PGJ$_2$), activate the nuclear hormone receptor peroxisome proliferator-activated receptor, isoform gamma (PPARγ, discussed in greater detail in Chapter 5) [1,2]. Until recently, this was the only known receptor for cyclopentenone PGs, resulting in

many proposals that attempted to associate PPARγ with the other major properties of cyclopentenone PGs, cell cycle arrest and apoptosis [3-6]. Though Δ12-PGJ_2 is a much lower affinity agonist for PPARγ than 15d-PGJ_2, and though PPARγ has never been associated with regulating any component of the proteasome pathway, we wanted to rule out the possibility that PPARγ activation by Δ12-PGJ_2 was the mechanism by which polyubiquitin was accumulating in cells. Data presented here demonstrate that the high affinity synthetic PPARγ agonist, troglitazone, which does not possess an α,β-unsaturated dienone, does not cause the accumulation of polyubiquitin in treated cells [Figure 4.1, lanes 2 – 7 vs. lane 1]. Under the same conditions, Δ12-PGJ_2 causes polyubiquitin accumulation, even at 20 μM [Figure 4.1, lane 8 vs. lane 1], a ten-fold lower concentration than troglitazone (200 μM).

Furthermore, the effects of PPARγ activation are mediated via gene activation, yet Δ12-PGJ_2 inhibits isopeptidase activity in whole cell lysates at concentrations similar to those seen in intact cells [Figure 4.2]. These whole cell lysates were sonicated during preparation, a process likely to result in the shearing, and thus inactivation, of all DNA and RNA. Therefore, these data suggest that the mechanism by which Δ12-PGJ_2 causes polyubiquitin accumulation is not mediated by PPARγ activation.

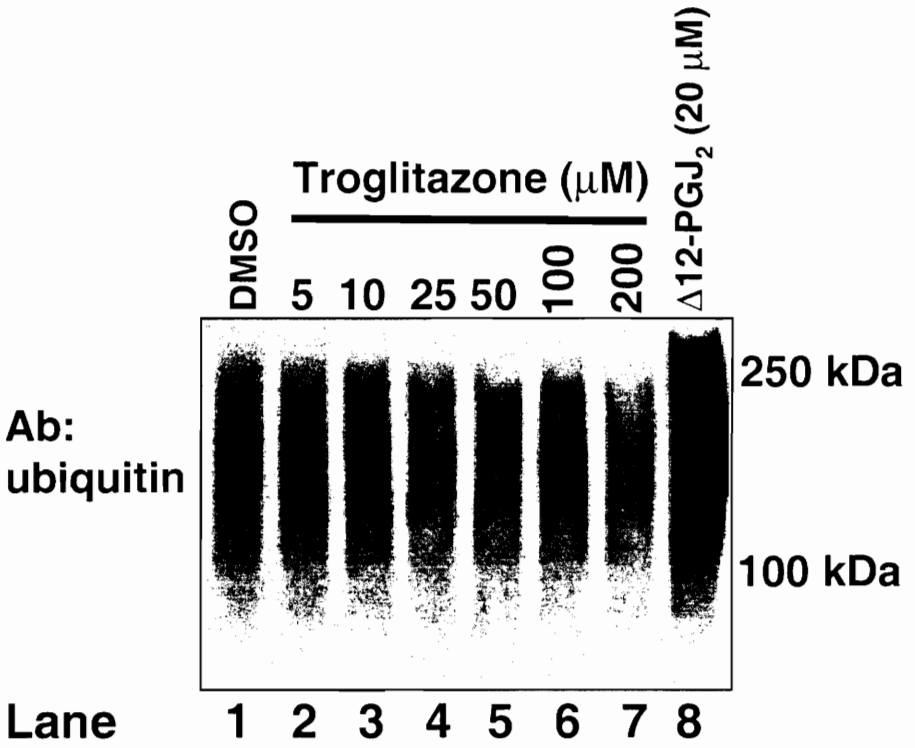


Figure 4.1. Effect of a PPARγ Agonist on Polyubiquitin Accumulation. RKO cells were treated with DMSO, the PPARγ agonist, troglitazone (5 – 200 μM), or Δ12-PGJ_2 (20 μM) for 6 hours. Cell lysate proteins were separated by polyacrylamide-gel electrophoresis, transferred to a PVDF membrane, and analyzed immunochemically with an antibody to ubiquitin to determine relative amounts of polyubiquitin.

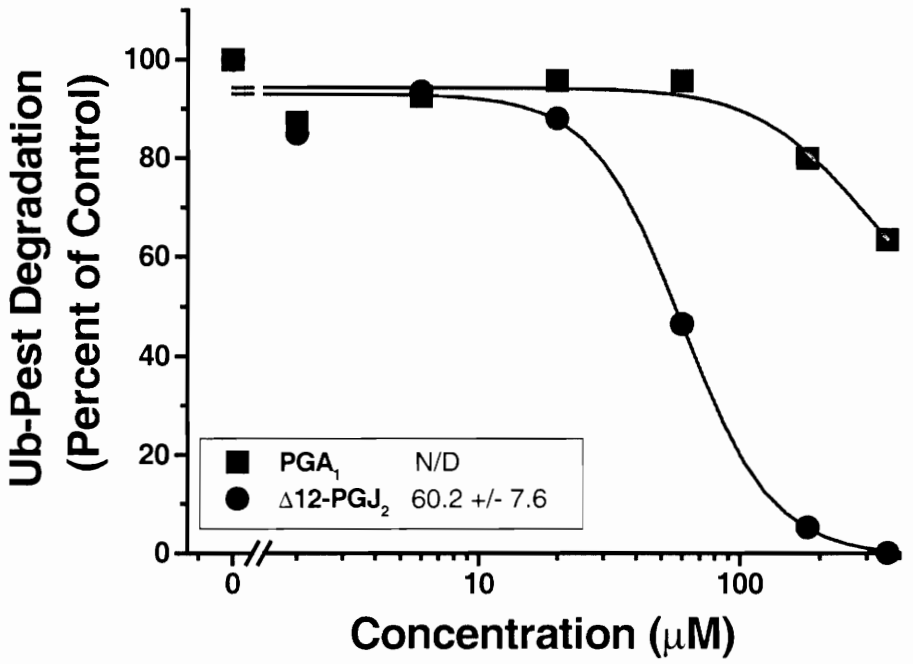


Figure 4.2. Effect of Cyclopentenone PGs on Isopeptidase Activity *In Vitro.* RKO cell lysates were treated with DMSO or 6 – 360 μM of PGA_1 or $\Delta 12\text{-}PGJ_2$ for 1 hour. Lysates were incubated with Ub-Pest, boiled in SDS loading buffer to stop the reaction, separated by polyacrylamide-gel electrophoresis, transferred to a PVDF membrane, and analyzed immunochemically with an antibody to ubiquitin to determine relative amounts of product formation (monoubiquitin). Monoubiquitin product was quantitated by densitometry and is represented as percent of control. Inset indicates ED_{50} (μM).

4.1.2. Δ12-PGJ$_2$ Does Not Cause Polyubiquitin Accumulation via Formation of Reactive Oxygen Species

Recent work demonstrated that cyclopentenone PGs cause the production of reactive oxygen species (ROS) in treated cells [7]. The authors claim that the increase in ROS caused polyubiquitin accumulation (via increased protein oxidation) and cell death, and that co-treatment with the antioxidant, α-tocopherol, reversed these phenotypes. However, they did not show data demonstrating a reversal of polyubiquitin accumulation. Therefore, I set forth to determine if generation of ROS played a role in the accumulation of polyubiquitin in Δ12-PGJ$_2$ treated cells. RKO cells were treated with either Δ12-PGJ$_2$ alone or in combination with α-tocopherol. No difference was observed in the accumulation of polyubiquitin with α-tocopherol co-treatment [Figure 4.3, lanes 2 and 3 vs. lane 1 and lanes 5 and 6 vs. lane 4], suggesting that ROS do not play a significant role in the accumulation of polyubiquitin in treated cells. I also did not observe any apparent effect of α-tocopherol co-treatment with regard to cell death (data not shown). However, it must be noted that the previous study was performed in a different cell type (neuronal cells), therefore, I cannot rule out the possibility that ROS generation plays a role in polyubiquitin formation in that model system, though again, they failed to show this data [7].

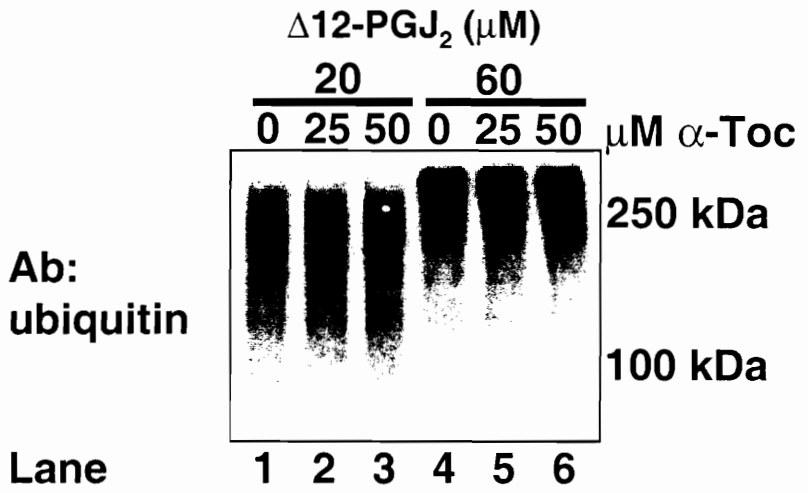


Figure 4.3. Effect of an Antioxidant on Polyubiquitin Accumulation. RKO cells were treated with 20 - 60 μM Δ12-PGJ_2 and 0 – 50 μM of the antioxidant, α-tocopherol, for 6 hours. Cell lysate proteins were separated by polyacrylamide-gel electrophoresis, transferred to a PVDF membrane, and analyzed immunochemically with an antibody to ubiquitin to determine relative amounts of polyubiquitin.

4.1.3. Δ12-PGJ_2 Inhibits Isopeptidase Activity Via a Covalent Mechanism

As the data in Figure 4.2 demonstrate, Δ12-PGJ_2, and to a lesser extent PGA_1, inhibit isopeptidase activity in sonicated cell lysates. Not only does this data strongly suggest that PPARγ plays a negligible role in polyubiquitin accumulation in treated cells, but, through the same reasoning, it suggests that no other transcriptional/translational mechanism plays a significant role in this phenotype. Furthermore, the data in Chapter 3, Figure 5, demonstrate that the inhibition of isopeptidase activity in treated lysates is, in the least, a high affinity binding reaction, as this inhibition cannot be reversed by dialysis. Combined, these data open up the possibility of a direct inactivation of isopeptidase(s) by Δ12-PGJ_2 in cells, consistent with cellular polyubiquitin accumulation. However, cell lysates are a heterogeneous mixture of cellular components, and therefore these data do not rule out the possibility that other isopeptidase regulatory components are the actual target(s) of Δ12-PGJ_2. Therefore I set out to determine if Δ12-PGJ_2 could inhibit the activity of a purified model isopeptidase.

Our data show that Δ12-PGJ_2 inhibits the activity of purified UCH-L3, consistent with direct inhibition of this isopeptidase [Figure 4.4]. It is noted that an exceptionally high concentration of Δ12-PGJ_2 (600 μM) is needed to achieve inhibition. However, this reaction is carried out in 10 mM DTT, which may effectively compete for accessible isopeptidase thiols. Also, this reaction was carried out at 4 °C, which may result in slowing the rate of reversibility between DTT and Δ12-PGJ_2. Furthermore, we have no data to suggest that UCH-L3 is the

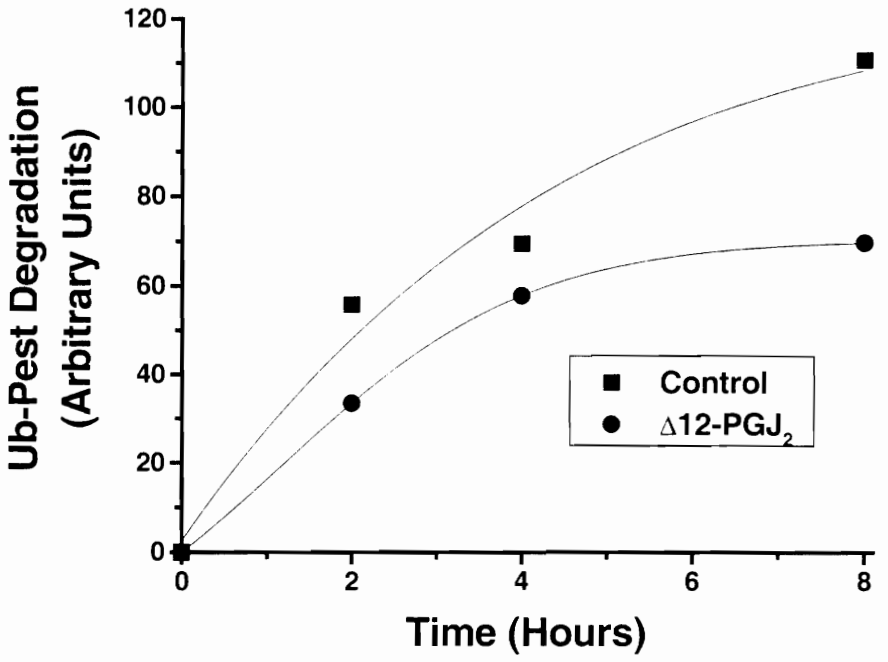


Figure 4.4. Effect of Δ12-PGJ_2 On Ubiquitin C-Terminal Hydrolase Activity *In Vitro.* UCH-L3 was incubated with DMSO or 600 μM Δ12-PGJ_2 for 3 hours at 4 °C, then incubated with Ub-Pest for 2 – 8 hours at 25 °C. The reaction was terminated by boiling in SDS loading buffer, and isopeptidase activity was determined as before. Monoubiquitin product was quantitated by densitometry and is represented as arbitrary units.

cellular target of Δ12-PGJ_2, therefore it may represent a low affinity target. These data, however, are consistent with covalent modification of isopeptidases, *in vitro*, suggesting that this mechanism may play a role in the inactivation of cellular isopeptidases, *in vivo*.

4.1.4. Δ12-PGJ_2 Causes Cell Death Via Inhibition of Global Cellular Protein Degradation

Cyclopentenone PGs are reactive molecules and are known to covalently modify multiple cellular proteins [8,9]. It is possible that other cellular targets, instead of isopeptidases, play a dominant role in the cytotoxicity of these compounds. I therefore set out to determine if isopeptidase inhibition is the prominent mechanism by which the model isopeptidase inhibitor, Δ12-PGJ_2, causes cell death. However, I have yet to determine the Δ12-PGJ_2-targeted isopeptidase(s) that is/are critical for maintaining proper cellular ubiquitin dynamics, so cellular 'rescue' experiments could not be performed by over-expressing any given isopeptidase. Instead I chose to manipulate a pathway we predict to be intricately linked with the isopeptidase inhibitor's mechanism of action, protein synthesis. My hypothesis, that Δ12-PGJ_2 inhibits ubiquitin isopeptidase activity, predicts that Δ12-PGJ_2 indirectly prevents proteasomal degradation, resulting in the inhibition of global cellular protein degradation. If inhibiting proteasomal degradation is what is causing cell death by Δ12-PGJ_2, then inhibiting protein synthesis should have a rescue effect on cells. This has in fact been demonstrated with the protein synthesis inhibitor, cycloheximide

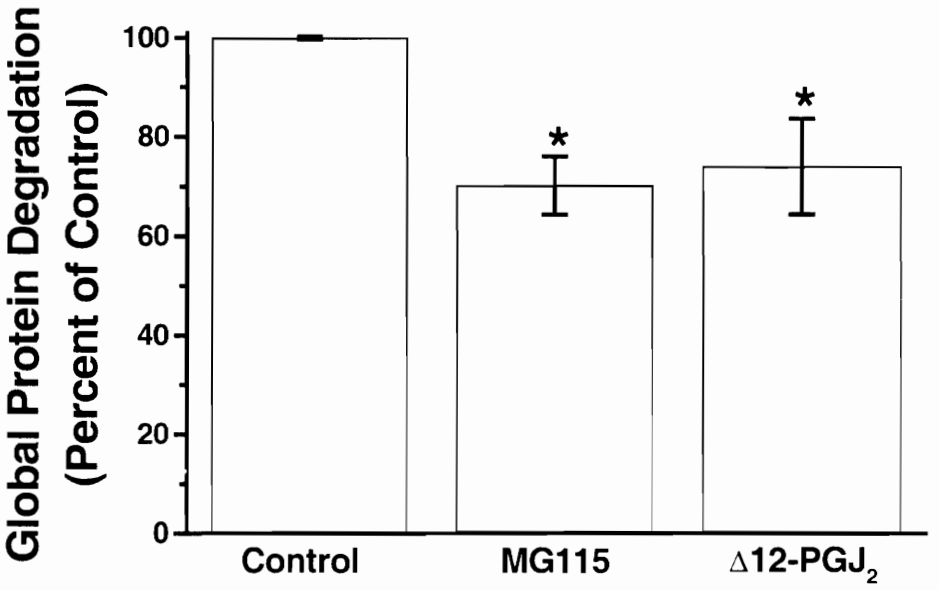


Figure 4.5. Effect of Proteasome and Isopeptidase Inhibitors on Global Protein Degradation. RKO cells were metabolically labeled with ^{35}S-methionine for 2 hours (pulse), then treated with DMSO, 20 μM MG115, or 60 μM Δ12-PGJ_2 for 2 hours in the absence of ^{35}S-methionine (chase). Cell lysates were subjected to trichloroacetic acid precipitation, separated into pellet (intact proteins) and supernatant (digested proteins), and quantitated by scintillation to determine the percentage of proteolysis (supernatant counts as a percent of total counts). Data are percent of control and are shown as the mean +/- S.D., n = 3 experiments, with statistical significance indicated (*, ANOVA, p <0.05).

(CHX) [10], however no explanation was given for this effect. If, as expected, CHX rescue is simply due to preventing the buildup of toxic levels of cellular proteins in the context of an inhibited ubiquitin-proteasome pathway, then rescue of polyubiquitin dynamics should accompany rescue from cell death by Δ12-PGJ_2.

First, I demonstrate that, similar to a catalytic-site inhibitor of the proteasome (MG115), inhibition of the ubiquitin-proteasome pathway by Δ12-PGJ_2 translates into inhibition of total cellular protein degradation [Figure 4.5]. Also, I show that both Δ12-PGJ_2 and MG115 cause cell death and, as expected, that co-treatment of cells with CHX prevents cell death under the same conditions [Figure 4.6]. It is unlikely that rescue from cell death is due significantly to cell cycle arrest caused by CHX, as this experiment is relatively short (7 hours). Furthermore, in agreement with the prediction that CHX's rescue effects have to do with restoring cellular protein homeostasis (i.e., protein synthesis $\cong$ protein degradation), CHX partially rescues the polyubiquitin accumulation and monoubiquitin depletion phenotypes in cells treated with 20 μM [Figure 4.7, lane 2 vs. lane 4] or 60 μM Δ12-PGJ_2 [Figure 4.7, lane 3 vs. lane 5]. Though MG115 does not cause polyubiquitin to accumulate under these conditions, therefore not allowing the observation of polyubiquitin rescue, the rescue effect is seen with regard to the depletion of monoubiquitin [Figure 4.7, lanes 6 and 7 vs. lanes 8 and 9]. Although not a definitive proof, these data are consistent with the hypothesis that cyclopentenone isopeptidase inhibitors cause cell death primarily by inhibiting cellular protein degradation.

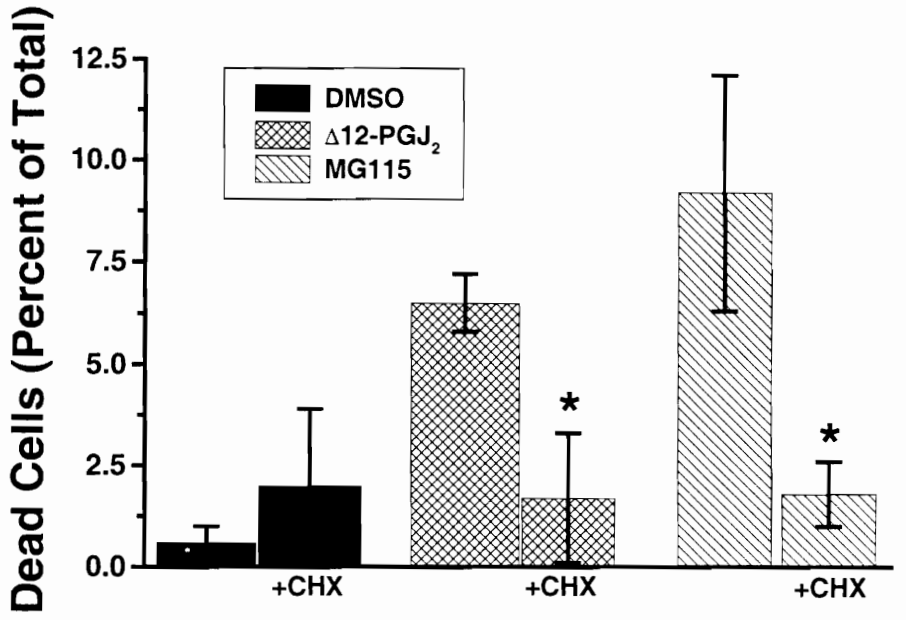


Figure 4.6. Effect of Protein Synthesis Inhibition on Cell Death Caused by Proteasome and Isopeptidase Inhibitors. RKO cells were treated with DMSO, 20 μM MG115, or 60 μM Δ12-PGJ_2 for 7 hours, +/- 10 μg/mL cycloheximide. Cells were trypsinized and stained with trypan blue to determine the number of non-viable cells. Data are percent of control and are shown as the mean +/- S.D., $n = 3$ experiments, with statistical significance indicated (*, ANOVA, $p < 0.05$).

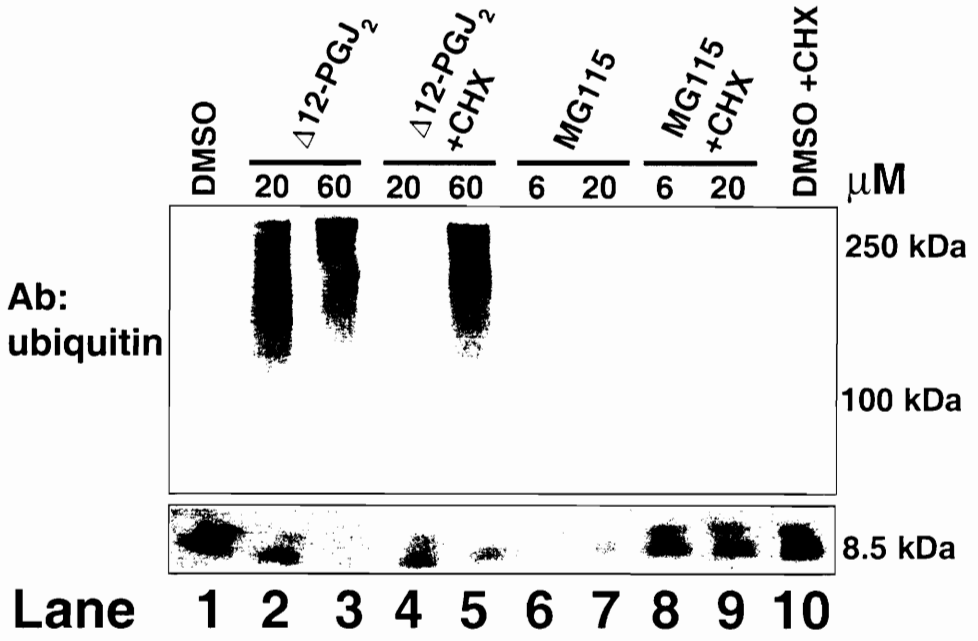


Figure 4.7. Effect of Protein Synthesis Inhibition on Polyubiquitin Dynamics. RKO cells were treated with DMSO, 6 μM to 20 μM MG115, or 20 μM to 60 μM Δ12-PGJ_2 for 7 hours, +/- 10 μg/mL cycloheximide. Cell lysate proteins were separated by polyacrylamide-gel electrophoresis, transferred to a PVDF membrane, and analyzed immunochemically with an antibody to ubiquitin to determine relative amounts of mono- and polyubiquitin.

4.2. Future Directions

4.2.1. Determine if Cell Death Proceeds Via ER-Stress Induced Activation of Caspase-12

One aspect of proteasome pathway that I am currently pursuing is with regard to how cells attempt to respond to toxic quantities of proteins and how this response can lead to cell death caused by proteasome pathway inhibitors. Others and I have observed that cyclopentenone PGs and catalytic-site proteasome inhibitors up-regulate cytosolic heat shock proteins, such as HSP70 [Figure 4.8, panel A.] [11-13]. Cyclopentenone PGs and catalytic-site proteasome inhibitors also up-regulate the resident heat shock protein of the endoplasmic reticulum (ER), GRP78 [Figure 4.8, panel B.] [13,14]. I have also observed that isopeptidase and proteasome inhibitors cause ER proliferation (data not shown), a sign that the ER is trying to cope with a large quantity of unfolded proteins. These results suggest that both of these types of proteasome pathway inhibitors cause the activation of the unfolded-protein response (UPR) of the ER. Cell death mediated via the UPR occurs by a novel apoptotic mechanism, only recently identified: activation of ER-specific caspase-12. The only identified activation of this caspase occurs due to ER stress; it then goes on to activate effector caspases, caspase-9 and caspase-3 [15]. Therefore, it will be interesting to determine if these proteasome pathway inhibitors activate caspase-12. Furthermore, it will be interesting to determine if inhibitors of heat shock proteins (e.g., the HSP90 inhibitor, geldanamycin) exacerbate cell death, and conversely, if promoters of protein re-folding (e.g., "chemical chaperones") rescue cells from cell death.

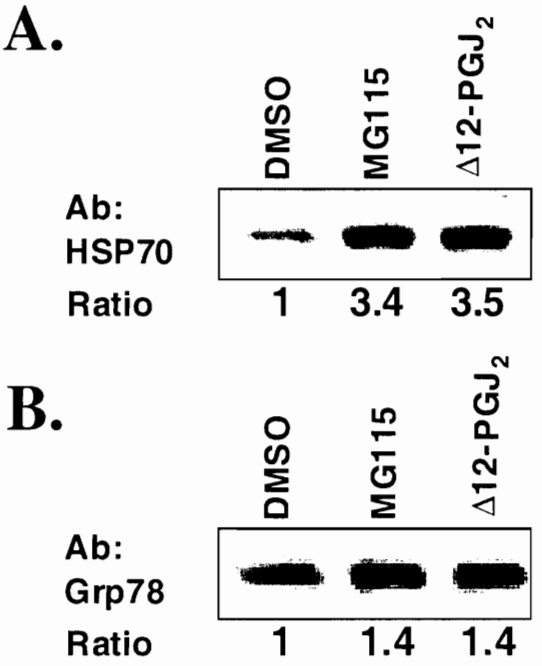
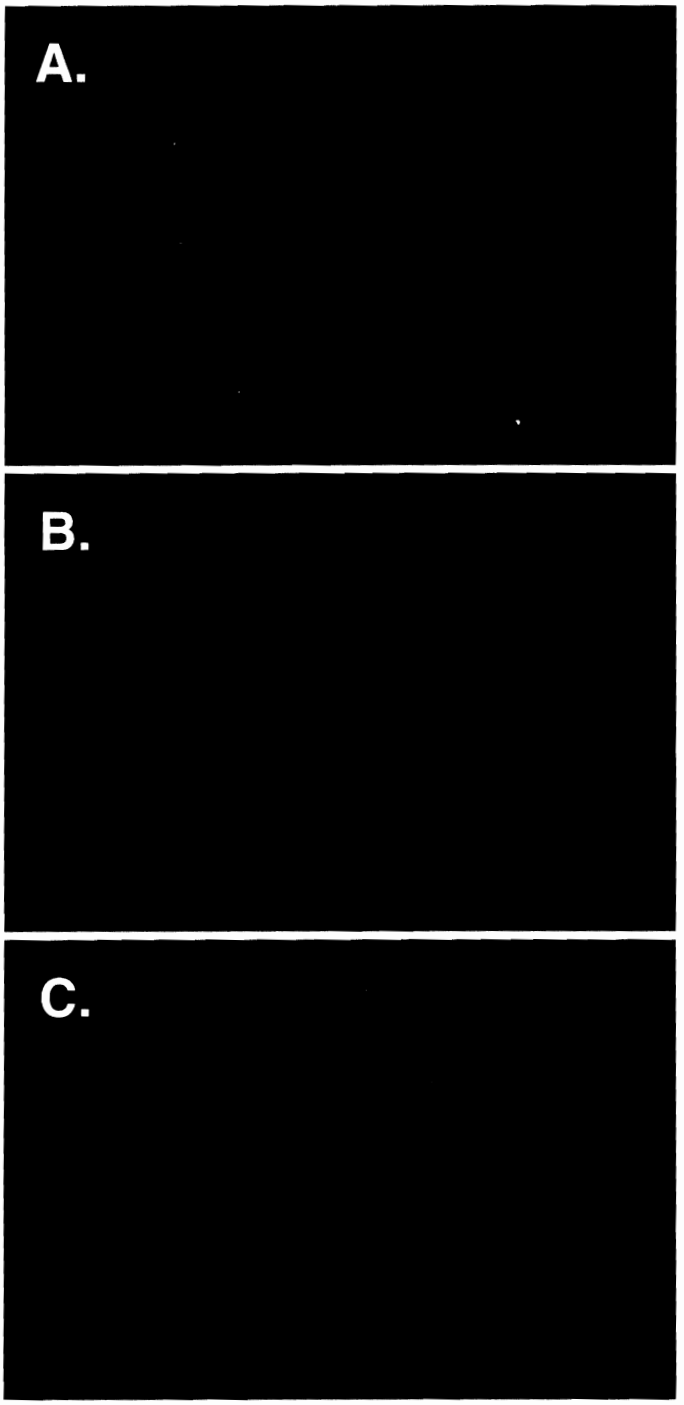


Figure 4.8. Effect of Proteasome and Isopeptidase Inhibitors On The Unfolded Protein Response. RKO cells were treated with DMSO, 20 μM MG115, or 60 μM Δ12-PGJ_2 for 6 hours. Cell lysate proteins were separated by polyacrylamide-gel electrophoresis, transferred to a PVDF membrane, and analyzed immunochemically with an antibodies to HSP70 (an inducible cytosolic heat shock protein), A., or GRP78 (BiP; an inducible ER heat shock protein), B. Protein bands were quantitated by densitometry to show the increase in HSP70 or GRP78, relative to control.

Figure 4.9. Effect of Proteasome and Isopeptidase Inhibitors on the Quantity of Cellular Lysosomes. RKO cells were incubated with DMSO, A., 20 μM MG115, B., or 60 μM Δ12-PGJ_2, C., for 4 hours. To detect cellular lysosomes and nuclei, cells were stained with 50 nM LysoTracker™ Red DND-99 and DNA was stained with 100 nM Hoechst 33342.


A.
B.
C.

Parallel to the ER/UPR observation, I have made the novel discovery that in cells in which the ubiquitin-proteasome pathway is inhibited, a compensatory increase in the number and/or size of acidic lysosomes occurs [Figure 4.9, panels B. and C. vs. A.; Δ12-PGJ_2, MG115, and DMSO, respectively]. This suggests that the cells are attempting to by-pass the proteasome pathway by activating alternate protein degradation pathways. This is interesting because knowing the pathways that lead to cell death may lead to effective combination therapies for PS-341, and may also aid in the early identification of patient populations that will be nonresponders.

4.2.2. Determine if Ubiquitin-like Isopeptidases are Inhibited by Δ12-PGJ_2

Other future work will include determining if other types of isopeptidases are inhibited by cyclopentenone PGs. There are approximately 11 ubiquitin-like molecules in eukaryotes that are used to covalently modify proteins in a manner analogous to ubiquitin (i.e., an isopeptide bond between the C-terminus of the ubiquitin-like monomer and a protein substrate lysine) [16,17]. Less is known about these modifications than about ubiquitination; however, it appears that most of them are used as monomers, and that none of them are used as targeting mechanisms for proteasomal degradation. In fact, some of these modifications are believed to antagonize ubiquitination by occupying lysine residues needed for polyubiquitin conjugation [18]. Interesting preliminary data suggest that de-sumoylation (deconjugation of Sumo-1) may be inhibited in cells treated with

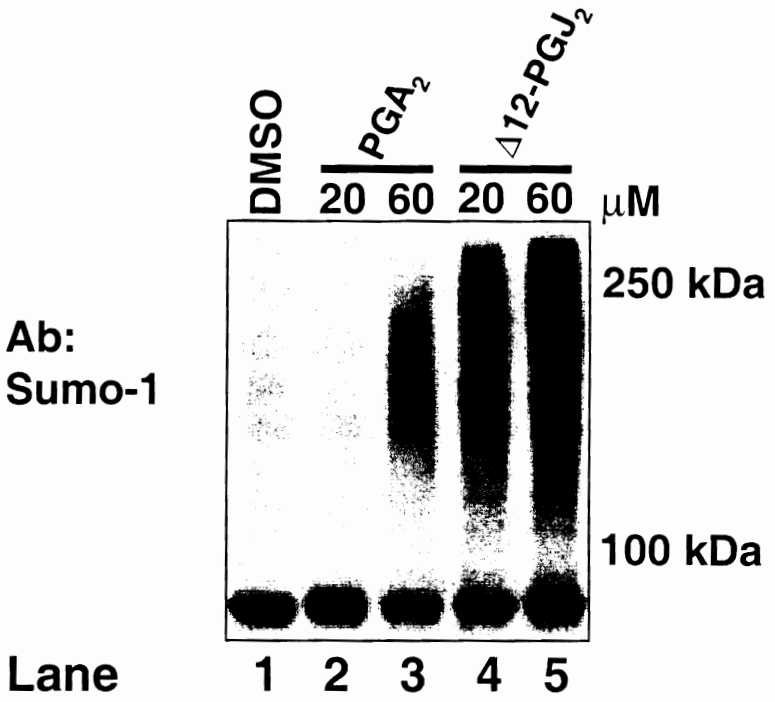


Figure 4.10. Effect of Cyclopentenone PGs on Sumo-1 Accumulation. RKO cells were treated with DMSO or 20 μM to 60 μM PGA_2 or $\Delta 12$-PGJ_2 for 6 hours. Cell lysate proteins were separated by polyacrylamide-gel electrophoresis, transferred to a PVDF membrane, and analyzed immunochemically with an antibody to Sumo-1.

cyclopentenone PGs [Figure 4.10., lanes 2 – 5 vs. lane 1]. What is of further interest is that, unlike with polyubiquitin accumulation, PGA_1 appears effective at causing the accumulation of high molecular weight sumoylated proteins under conditions in which polyubiquitin does not accumulate. While these PGs have differences in potency with regard to cell death and proteasome pathway inhibition, they have similar potency with regard to the inhibition of several transcription factors, such as p53 [19]. It is believed that p53 levels are regulated by sumoylation, therefore it will be interesting to determine if Sumo-1 plays a role with regard to cyclopentenone PG-mediated inactivation of p53 and/or other transcription factors.

4.2.3. Identify Isopeptidase(s) Inhibited by Δ12-PGJ_2

An obvious future direction is to attempt to identify the isopeptidase(s) that PGs inhibit. Over-expressing this enzyme in cells would allow for more meaningful 'rescue' experiments to be performed, and more detailed mechanism of action data to be collected. Furthermore, identification would be a first step in the synthesis of specific inhibitors to isopeptidases, which may have more favorable pharmacological profiles in model organisms than Δ12-PGJ_2.

4.2.4. Model Chronic Inflammation with Low Concentrations of Δ12-PGJ_2 to Determine Long-term Effects

Lastly, it would be interesting to determine to what degree chronic inflammation plays a role in altering the kinetics of the proteasome pathway.

Although most of this work has centered around relatively high concentrations of PGs for short time periods (e.g., 60 μM of Δ12-PGJ_2 for ≤ 12 hours; Chapter 2, Figure 5), I have observed that at lower concentrations for longer time periods (e.g., 6 μM of Δ12-PGJ_2 for ≤ 24 hours; Chapter 3, Figure 4), polyubiquitin accumulates. This is interesting because it takes ~3 weeks for colon cells to migrate from the base of the colon crypt to the top, where they are sloughed off. The longest of the experiments in this work was 48 hours, so since pharmacology is a function of time and concentration, it will be interesting to see what effect long-term treatment with very low concentrations of PGs will have on proteasome pathway inhibition.

4.3. Materials and Methods

4.3.1. Materials

I used Δ12-PGJ_2 and PGA_1 (Cayman Chemicals, Ann Arbor, MI); MG115 (Peptides International, Louisville, KY); Troglitazone and UCH-L3 (Biomol, Plymouth Meeting, PA); α-tocopherol, cycloheximide, trichloroacetic acid, and chloroquine (Sigma-Aldrich, St. Louis, MO); EasyTag 35-S Protein Labeling Mix (NEN Life Sciences, Boston, MA); trypan blue (Invitrogen Corporation, Carlsbad, CA); LysoTracker™ Red DND-99, SlowFade® Light Antifade Kit, and Hoechst 33342 (Molecular Probes, Eugene, OR); complete protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN); enhanced chemiluminescence reagents (Amersham Pharmacia, Piscataway, NJ); antibodies directed against ubiquitin and Sumo-1 (Zymed Laboratories, Inc., San Francisco,

CA), GRP78 (BD Biosciences, San Jose, CA), HSP70 (Stressgen Biotechnologies, Inc., San Diego, CA), horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Ub-PEST (gift of Dr. Martin Rechsteiner, Department of Biochemistry, University of Utah).

4.3.2. Cell Culture

I used RKO colon cancer cells (gift from Dr. Mark Meuth, Institute for Cancer Studies, University of Sheffield, Sheffield, U. K.). RKO cells were maintained in DMEM (supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin and streptomycin, and 10% (v/v) FBS) in a humidified incubator with 5% CO_2.

4.3.3. Immunochemical Detection of Proteins

I removed the medium and lysed cells in 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM EDTA with 0.1% SDS, 0.1% deoxycholate, 1× complete protease inhibitor mixture. Protein concentration was measured by the Bradford method. Equal portions of the total cell lysate from each sample (12.5 μg of protein) were fractionated by SDS-PAGE and transferred to poly(vinylidene difluoride), which was blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline [20 mM Tris·HCl, pH 7.5, 100 mM sodium chloride, 0.1% (v/v) Tween 20]. Proteins were detected immunochemically by using primary antibodies directed against Sumo-1 (1:1000), GRP78 (1:250), HSP70 (1:1000), or ubiquitin (1:1000), followed by horseradish peroxidase-conjugated secondary antibodies (1:4000). Antigen-

antibody complexes were detected with enhanced chemiluminescence reagents. Lastly, gels were scanned and band intensities were quantified using Kodak 1D Image Analysis Software.

4.3.4. *In vitro* Ubiquitin Isopeptidase Activity Assays

I measured cellular isopeptidase enzymatic activity with ubiquitin-PEST (Ub-PEST), a full-length ubiquitin molecule with an 18 amino acid c-terminal peptide extension (total mass = 10.5 kDa). Ubiquitin isopeptidases specifically cleave the 18 amino acid peptide extension, releasing full-length ubiquitin (8.5 kDa). Briefly, we lysed RKO cells in 50 µL of 25 mM HEPES, 5 mM EDTA, 0.1% CHAPS, 5 mM ATP, pH 7.5. I adjusted the protein concentration of each sample to 0.3 mg/mL and incubated with 0-360 µM PGA_1 or $\Delta 12\text{-}PGJ_2$ for 1 hour. Treated lysates were incubated with 50 µg/mL Ub-PEST for 45 minutes at 25 °C. Under these conditions Ub-PEST hydrolysis occurs at a linear rate. I mixed 20 µL samples with 20 µL 2X Laemmli buffer, boiled briefly, and fractionated by SDS-PAGE. Last, isopeptidase activity was monitored by determining the extent of product (8.5 kDa ubiquitin) formation.

4.3.5. UCH-L3 Activity Assay

I measured the enzymatic activity of ubiquitin c-terminal hydrolase-L3 (UCH-L3) with ubiquitin-PEST (Ub-PEST) as above. Briefly, I pre-incubated 10 nM UCH-L3 in 10 mM DTT, 0.1 mg/mL BSA, and 50 mM Tris, pH 7.4 for 15 minutes. UCH-L3 was then incubated with either DMSO or 60 µM $\Delta 12\text{-}PGJ_2$ for

3 hours at 4 °C, followed by incubation with 50 μg/mL Ub-PEST for 2 through 8 hours at 25 °C. Under these conditions Ub-PEST hydrolysis occurs at a linear rate. I mixed 20 μL samples with 20 μL 2X Laemmli buffer, boiled briefly, and fractionated by SDS-PAGE. Last, isopeptidase activity was monitored by determining the amount of product (8.5 kDa ubiquitin) formation.

4.3.6. Protein Degradation Assay

I plated 1.5 x 10^5 RKO colon cancer cells into each well of a 12 well plate and incubated overnight. Each well was aspirated, washed twice with medium lacking methionine, and filled with 0.25 mL of media lacking methionine but having 200 μCi of ^{35}S-methionine. Cells were incubated with ^{35}S-methionine for 2 hours, aspirated, washed twice with cold phosphate buffered saline (PBS), and incubated with 0.5 mL of media containing methionine, chloroquine (200 μM; to inhibit lysosomal degradation), and desired treatment for 2 hours. Wells were again aspirated and washed twice with PBS. Cells were then lysed in 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM EDTA, 0.1 percent SDS, 0.1 percent deoxycholate, and 1X complete protease inhibitors. Intact protein was precipitated from the cell lysates with 10 percent trichloroacetic acid (TCA), incubated on ice for 30 minutes, and subjected to centrifugation (15, 000 x g, 10 minutes). TCA soluble peptides (supernatant) were then separated from insoluble, intact peptides (pellet) for analysis by scintillation. Percent soluble peptides was determined with the following equation: soluble counts/(soluble counts + pellet counts) X 100.

4.3.7. Trypan Blue Exclusion Assay

I plated 1.5 x 10^5 RKO colon cancer cells into each well of a 12 well plate and incubated overnight. Cells were treated as indicated for 7 hours. The media from each well was transferred to a microcentrifuge tube, and the cells from each well were trypsinized, placed into the corresponding microcentrifuge tube, and subjected to centrifugation (450 x g, 3 minutes). The media was aspirated from each tube, leaving approximately 100 µL left in each tube, and 100 µL of trypan blue dye was added. Cells were pipetted into a hemocytometer and the percentage of blue-stained cells (dead cells) was determined. Approximately 400 cells were counted per treatment.

4.3.8. Fluorometric Histochemistry

I plated RKO cells on 8-well chamber slides at 2.5 $\times 10^4$ cells per well in standard medium. Cells were incubated with DMSO, 60 µM Δ12-PGJ_2, or 20 µM MG115 for 4 hours. Cellular lysosomes were stained with 50 nM LysoTracker™ Red DND-99 and DNA was stained with 100 nM Hoechst 33342 for 1 hour. Cells were washed with cold STP buffer, fixed in a 10 percent formalin solution, and washed 2× with STP buffer. To protect from photobleaching, the cells were mounted in ProLong® Antifade reagent prior to fluorescence microscopy.

4.3.9. Statistics

We used analysis of variance (ANOVA) for statistical calculations.

4.4. References

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CHAPTER 5

MODEL FOR PROSTAGLANDINS IN APOPTOSIS

5.1. Background: Cyclopentenone PGs in Apoptosis

Prostaglandins [Figure 5.1] are molecules that are derived from the conversion of arachidonic acid to PGH_2 by the cyclooxygenase enzymes, COX-1 and COX-2 [1]. COX-1 is a constitutively expressed enzyme, the function of which is necessary for cellular homeostasis. COX-2, however, is an inducible isoform, the expression of which is significantly elevated at sites of inflammation and in cancer [1]. Many of the PGs have physiological effects on smooth muscle via their properties as ligands for G-protein coupled receptors [2]. The spontaneous, and albumin catalyzed, dehydrations of PGE_2 and PGD_2 lead to the formation of the cyclopentenone PGs, PGA_2 and Δ12-PGJ_2, respectively [3,4]. Cyclopentenone PGs do not act via G-protein coupled receptors and do not have effects on smooth muscle. However, these PGs have gained activity that the non-cyclopentenone PGs do not generally possess: relative potency with regard to induction of apoptosis [5]. Despite many years of study, no cohesive data set has led to a definitive mechanism of action with regard to induction of apoptosis by cyclopentenone PGs [Figure 5.2].

Due to the apoptosis-promoting activity of the cyclopentenone PGs, there is a great deal of interest in finding their mechanism of action. Adding to this interest is the fact that they appear to cause apoptosis by a novel mechanism, since cell lines that have developed resistance to standard chemotherapeutics remain sensitive to cyclopentenone PGs [6,7]. Furthermore, cyclopentenone PGs appear to sensitize cells to certain clinically useful chemotherapeutics, in particular, the DNA damaging agents, such as cisplatin [8]. Given that DNA

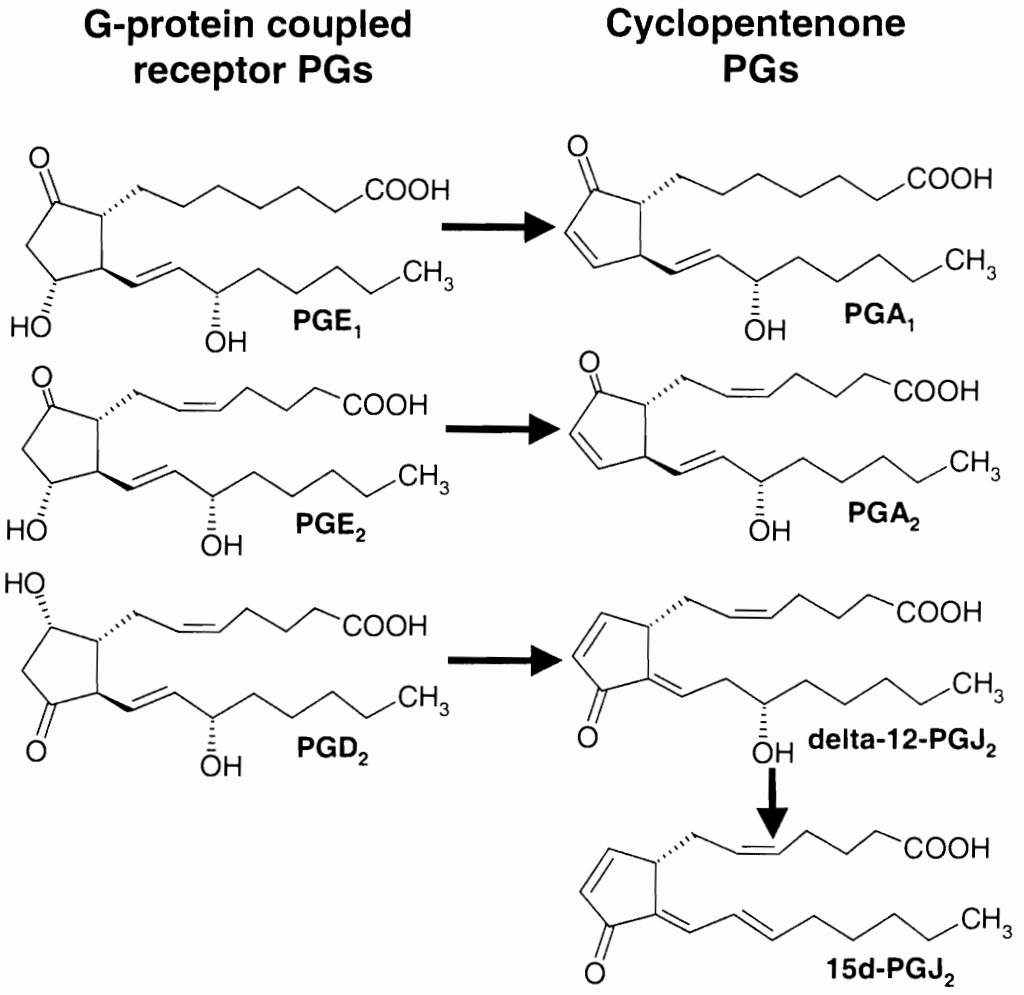

Figure 5.1. Representation of Various Prostaglandins. Shown are a representation of prostaglandins (PGs) that have effects on G-protein coupled receptors (left column). Arrows indicate spontaneous and/or albumin catalyzed dehydration that lead to the formation of the cyclopentenone PGs (right column). Cyclopentenone PGs are relatively more potent than their respective noncyclopentenone parent compounds at inducing apoptosis.

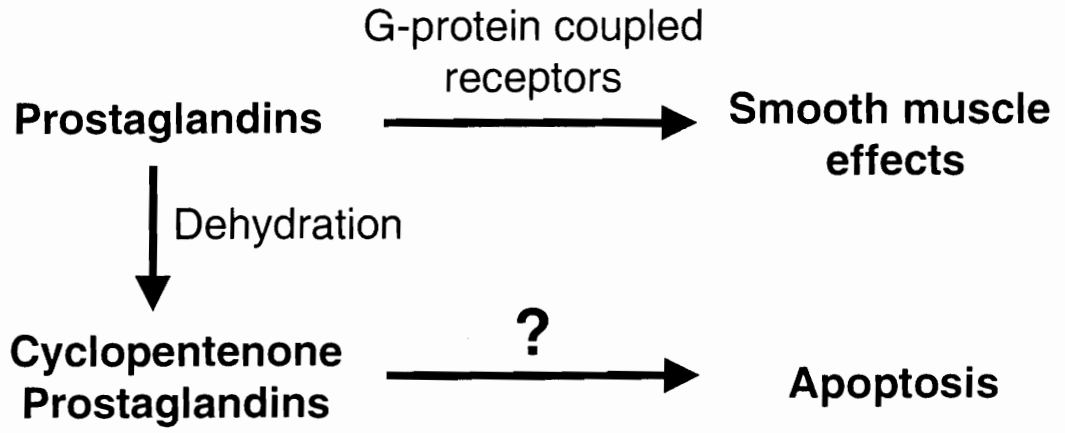

Figure 5.2. Previous Model for the Cellular Effects of PGs. Shown is a schematic representing the effects of the two major classes of PGs, smooth muscle effects and apoptosis. The role of various noncyclopentenone PGs as activators of G-protein coupled receptors is well established. No coherent rationale exists explaining why cyclopentenone PGs cause apoptosis.

damaging agents are the mainstay of clinical treatment regimens, enhancing their activity *in vivo* may be useful in treating cancers that acquire resistance to DNA damaging agents or cancers typically not amenable by treatment with DNA damaging agents.

The interest in discovering the mechanism of action for the cyclopentenones also stems from more practical considerations. When administered to whole organisms, the half-life of these compounds is relatively short (< 10 minutes *in vitro* and *in vivo*) [9,10]. Therefore, finding the relevant target(s) for these compounds may lead to the development of more specific compounds having more favorable pharmacological profile (i.e., greater specificity, bioavailability, potency, etc.).

5.2. Previously Stated Hypotheses

5.2.1. PPARγ Activation Causes Apoptosis

The great interest in the cyclopentenone PGs, due to the promising apoptotic effects seen in model cell lines, has generated several hypotheses as to their mechanism of action. The most prominent of these hypotheses regards the relationship of PGs of the J-series, in particular 15-deoxy-Δ12,Δ14-PGJ_2 (15d-PGJ_2), as agonists of the peroxisome proliferator-activated receptor, isoform gamma (PPARγ). PPARγ is a nuclear hormone receptor that activates genes that regulate adipogenesis. 15d-PGJ_2 is the highest affinity, naturally occurring cellular ligand known for this receptor [11,12]. It has been suggested that activation of PPARγ is the mechanism by which Δ12-PGJ_2 causes cell cycle arrest and

induces apoptosis [5,13-15]. However, several problems exist with this model. Δ12-PGJ_2 is a much lower affinity ligand for PPARγ, yet in it is as potent at inducing apoptosis as 15d-PGJ_2 [16,17]. 15d-PGJ_2 causes apoptosis in cells that do not express the PPARγ receptor [18]. PPARγ antagonists have no effect on Δ12-PGJ_2-induced apoptosis [19,20]. Finally, synthetic PPARγ agonists (e.g. thiazolidinediones), which have much higher affinity for PPARγ than does 15d-PGJ_2, are not as potent at inducing apoptosis as PGs of the J-series, and in fact do not cause apoptosis themselves unless at much higher concentrations than would be necessary to saturate PPARγ binding [20]. Interestingly, one recent study demonstrated that cyclopentenone prostaglandins induced activation of PPARα and PPARδ, *without* binding to these receptors, suggesting these receptors are not activated directly by the PGs, but instead by some unknown alternative mechanism [21]. This opens up the possibility of alternative mechanisms for the activation of PPARγ by Δ12-PGJ_2.

5.2.2. Heat Shock Protein Induction Causes Apoptosis

One of the original phenotypes observed in cells treated with cyclopentenone PGs was the induction of heat shock proteins (HSPs) [22]. This prominent phenotype has led some to hypothesize that the induction of HSPs is the causal factor for PG-induced apoptosis [23]. Indeed, PGs of the J-series mimic heat shock with regard to induction of heat shock proteins and G1 arrest; however, these studies never demonstrate a causal relationship between the HSPs and G1 arrest. Heat shock causes the unfolding of proteins by denaturation,

therefore, any number of factors may be responsible for cell cycle arrest. In fact, the role of HSPs as molecular chaperones that aid in the refolding of denatured proteins supports the idea that HSP induction is consequence and not a cause. Furthermore, one study demonstrated that HSP overexpression actually protects cells from PG-induced apoptosis [24].

5.2.3. Induction of Important Regulatory Proteins Causes Apoptosis

Many of the remaining hypotheses regarding cyclopentenone PG-induced G1 arrest and apoptosis center around the observed accumulation of various proteins. For example, p53 has been shown to be up-regulated in a number of cell lines due to cyclopentenone PG treatment [25,26]. Since p53 protein levels are regulated not by transcription but by degradation, the general assumption in these studies is that p53 is accumulating due to signaling events (analogous to DNA damage signals) thus activating it as a transcription factor. Supporting this evidence, $p21^{Waf1}$, a target gene of p53-mediated transcription, is often seen to be up-regulated at the protein level [26-28]. However, no studies have demonstrated that the accumulated p53 is actually active as a transcription factor. In fact, as our work has shown, p53 in not active as a transcription factor despite its accumulation [29]. Furthermore, the effects of cyclopentenone PG treatment (e.g. G1 arrest, p21 accumulation, and apoptosis) are similar in cells that lack p53 [30]. Lastly, other than demonstrating the accumulation of these proteins, no evidence is provided suggesting *why* they are accumulating.

5.3. Proposed Model: Inhibition of Protein Degradation Causes Apoptosis

The model proposed herein [Figure 5.3] is one in which cyclopentenone prostaglandins (with PGs of the J-series being relatively more potent than PGs of the A-series) inhibit a ubiquitin isopeptidase(s) that is essential for maintaining the proper intracellular balance between monoubiquitin and polyubiquitin. The disruption of this balance by PGs leads to an accumulation of polyubiquitin, and subsequent depletion in monoubiquitin [Figure 3.4], and a decrease in global cellular protein degradation by the proteasome pathway [Figure 4.5], similar to catalytic site proteasome inhibitors. If correct this model would reconcile many of the observations made in cells treated with cyclopentenone PGs.

First, the inhibition of protein degradation is predicted to cause an accumulation of many normal and misfolded proteins that would normally be targeted for degradation. This could account for the accumulation of p53 (normal and misfolded) and $p21^{Waf1}$ observed by our group and others [25-28]. Also, we speculate that protein accumulation could help explain the proposed alternate mechanism for PPARγ activation. PPARγ activation involved its heterodimerization with the retinoic acid X receptor, isotype alpha (RXRα) [31]. RXRα is degraded by the proteasome pathway, therefore its accumulation may result in increased binding and activation of PPARγ [32].

Second, the model I propose helps reconcile all of the various cyclopentenone PG-induced stress responses. The accumulation of

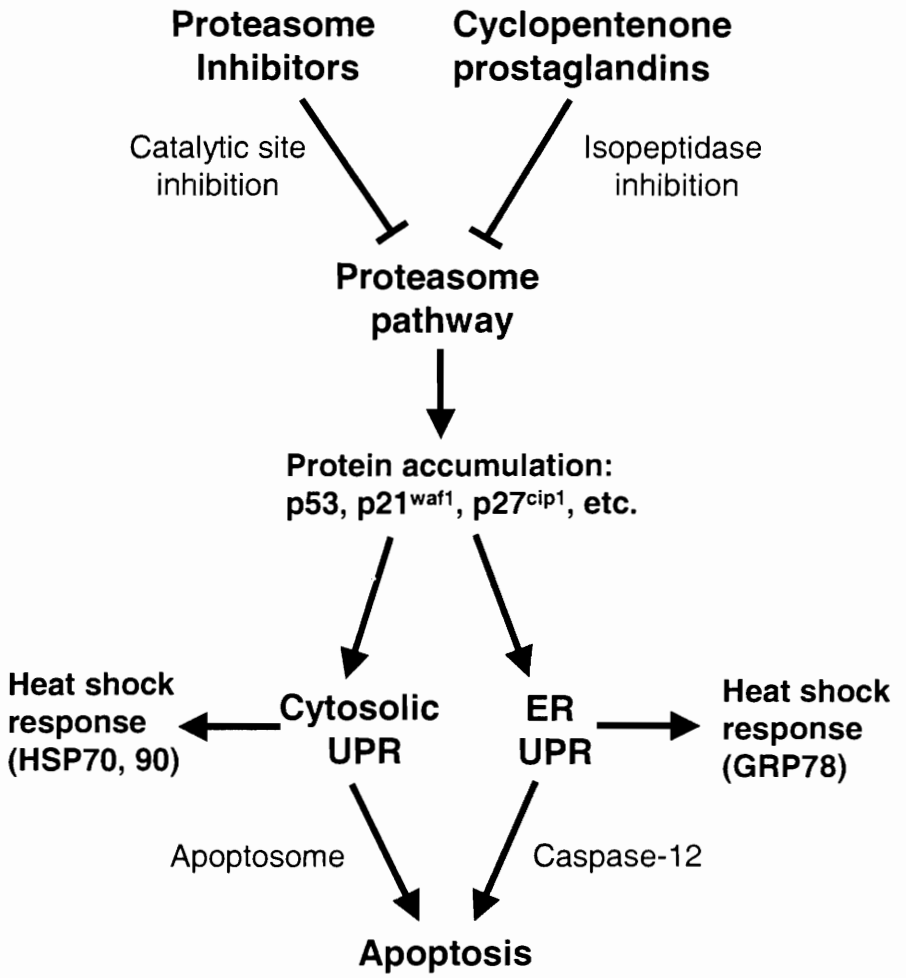

Figure 5.3. The Proposed Model for Cyclopentenone PG-Induced Apoptosis. Shown is a schematic representing the model proposed herein for cyclopentenone PG-induced apoptosis. First, cyclopentenone PGs inhibit the proteasome pathway, via isopeptidase inhibition. Similar to the effect of catalytic-site proteasome inhibitors, this causes the accumulation of normal and unfolded proteins to toxic levels, resulting in the apparent up-regulation of specific proteins and the induction of the heat-shock response. This model proposes that the inability of cells to degrade accumulating unfolded proteins results in cytosolic and ER unfolded protein response (UPR) activation and apoptosis.

deranged/unfolded proteins triggers the heat shock response of the cytoplasm and the unfolded protein response (UPR) of the endoplasmic reticulum (ER). Genes up-regulated by these responses include the cytoplasmic HSPs, HSP70 and HSP90, the ER resident HSP, GRP78 (BiP), ubiquitin-B, and ubiquitin-C [33]. Supporting that the inhibition of the ubiquitin-proteasome pathway by cyclopentenone PG could be cause these effects, catalytic site inhibitors of the proteasome cause up-regulation of the cytoplasmic and ER HSPs and ubiquitin [34].

Last, this model would help explain why the protein synthesis inhibitor, cycloheximide, protects cells from cyclopentenone PG-induced apoptosis. Due to the mechanism of action of cycloheximide, this effect has always been correctly assumed to be the result of inhibiting nascent protein synthesis [35]. However, some studies have attempted to link this phenomenon with the inhibition of the synthesis of a particular protein [36]. The model I propose suggests a more global explanation in which cycloheximide simply prevents the synthesis of proteins that would only add to a growing pool of deranged/unfolded proteins. In this way cycloheximide may delay cytoplasmic ER stress cause by cyclopentenone PGs. Supporting this hypothesis are data presented here [Figure 4.6] demonstrating that cycloheximide also protects cells from the effects of catalytic site inhibitors of the proteasome.

In summary, cyclopentenone PGs share in common with catalytic-site proteasome inhibitors the following properties: disruption of ubiquitin homeostasis, inhibition of protein degradation, accumulation of a number of proteins regulated by the proteasome pathway, up-regulation of heat shock

proteins, and rescue from cell death by cycloheximide co-treatment. Therefore, many of the observations previously made regarding the effects of cyclopentenones PGs can be reconciled with our model that these compounds inhibit the proteasome pathway via inhibition of ubiquitin isopeptidase activity [Figure 5.3].

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